Issued October 2, 1912.

U. S. DEPARTMENT OF AGRICULTURE,

BUREAU OF ANIMAL INDUSTRY.—BULLETIN 150.

A. D. MELVIN, CHIEF OF BUREAU.

THE BACTERIOLOGY OF CHEDDAR CHEESE.

BY

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Bacteriologist, Wisconsin Agricultural Experiment Station,

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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., May 24, 1912.

Sir: I have the honor to transmit for publication in the bulletin series of the bureau the accompanying manuscript entitled "The Bacteriology of Cheddar Cheese," by Messrs. E. G. Hastings and E. B. Hart, of the Wisconsin Agricultural Experiment Station, and Miss Alice C. Evans, of the Dairy Division of this bureau.

Cooperative work on the factors concerned in the ripening of Cheddar cheese has for several years been carried on at Madison, Wis., between the Dairy Division and the Wisconsin Experiment Station, and this bulletin is one of a series in which the results of the work are detailed. Previous experiments have already been described in Bulletin 122 of the Bureau of Animal Industry, entitled "Factors Controlling the Moisture Content of Cheese Curds," and in Bulletins 7 and 11 of the Wisconsin Station.

In connection with the present study credit is due to Mr. L. D. Bushnell, now with the Kansas Agricultural Experiment Station, and Mr. Alfred Larsen, of the North Dakota State Hygiene Laboratory, who were formerly bacteriologists in the Dairy Division and while thus engaged prepared a portion of the data presented in this bulletin.

Respectfully,

A. D. Melvin,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

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THE BACTERIOLOGY OF CHEDDAR CHEESE.

INTRODUCTION.

The rôle of microorganisms in the preparation and ripening of the various kinds of cheese is a problem that has attracted the attention of bacteriologists since the beginning of the science and since the establishment of the importance of microorganisms in all decomposition processes. From the same raw materials—cow's milk, salt, and rennet, which is an extract of the fourth stomach of the young calf—a great number of varieties of cheese are prepared. These cheeses differ not only in texture, but more especially in flavor and aroma. From what is known of the importance of microorganisms in the preparation of the products of the fermentation industries it is evident to one who gives consideration to the question that undoubtedly microorganisms are as essential in the making of cheese as they are known to be in the preparation of wine, beer, and other products.

In the making of wine and beer the desired changes are produced by a single form of life, the true yeasts, and in the preparation of any desired type of product attention need only be directed as far as the causal organism is concerned in order to insure the presence of the particular variety of yeast that has been found by experience to form a product that has the desired properties as well as to insure the absence of harmful forms. In the preparation of still other products of the fermentation industries not only a single form of life is essential to produce the desired changes, but two or more are needed, one of which may work on a certain constituent of the raw material, the other on another, or the one may serve to make conditions favorable for the growth of the second, usually by the preparation of suitable food.

It is evident that as the number of kinds of microorganisms that are needed to produce the desired changes in any product increases, the complexity of the problem of demonstrating the rôle of each of them is also greatly increased, and it may become most difficult to prove even whether any particular organism is absolutely essential in the preparation of the product, to say nothing of demonstrating its exact rôle. It is not to be supposed that a single form of life is

responsible for the complex chemical changes that occur in the ripening of any particular variety of cheese, but rather that a considerable number will be involved.

The fermentation industries include those in which microorganisms are essential for the production of the desired decomposition changes. In all such industries the quality of the product will depend on the raw material and the organisms that are used in the decomposition of that material. The preparation of cheese has not until recent years been classed as a fermentation industry, although it is one in which microorganisms play a dominant rôle. Here, as in all other similar industries, if control of the quality of the product is to be obtained, knowledge must be gained concerning the essential biological agents. In this paper are presented a summary of the present knowledge of the bacteriology of Cheddar cheese and the results of a detailed study of a number of cheeses.

THE BACTERIAL FLORA OF MILK.

In the case of many of the products of the fermentation industries the essential microorganisms are contained in the raw materials, and the development of the methods of preparation of the products have been wholly empirical. Especially is this true of certain kinds of cheese, the methods of manufacture of which are in some cases centuries old. Even in the case of cheese to which at some stage in manufacture material containing certain microorganisms is added the methods of manufacture antedate the science of bacteriology.

The sources from which microorganisms enter the milk are much the same in all parts of the world. Any natural source, such as the interior of the udder, the dust from the animal, or even the utensils, will always furnish certain definite types of microorganisms. By this is not meant that the flora of milk will always include these definite types and no others, but that certain forms will always be present. That this is true is shown by the fact that a sample of ordinary market milk, if stored at temperatures ranging from 60° to 90° F., will undergo a definite sequence of decomposition changes with almost unerring certainty. In this sequence three main groups of microorganisms are prominent; first, the acid-forming bacteria that change the milk into an acid, semisolid mass, favorable for the development of the mold that is characteristic of milk—*Oidium lactis*. This mold, by its gradual destruction of the acid and the establishment of an alkaline reaction, makes it possible for the putrefactive organisms to develop. When the environment is changed this sequence of microbial life in the milk is also changed. For example, milk may be kept at such low temperatures that the acid-forming bacteria can not grow and the putrefactive bacteria appear first.

It is thus proper to speak of a characteristic milk flora. By some it is believed that this flora is so constant that fermentations that depart from the normal are to be looked upon not as due to the introduction of other forms of microorganisms, but most often to changes in the properties of the organisms or to changes in environment that favor the particular organisms which produce the abnormal fermentation. Slight changes in the composition of the milk may also have a determining influence.

As has been stated, the microorganisms necessary for the preparation of most kinds of cheese are contained in the milk. Whether one form or the other is to develop in the cheese depends upon the environment established by the cheese maker. The manufacture of cheese is thus a problem in the ecology of microorganisms.

DIFFERENCES IN COMPOSITION OF CHEESE.

The differences due to the methods of manufacture result in differences in composition of the cheese at the time it begins to undergo the ripening process, which is to determine whether the cheese is to be classed as one kind or another. The differences in composition of the fresh cheese are very largely in the amount of moisture present. Since the water that is left in the cheese is whey, carrying in solution sugar and other constituents of milk, the effect of differences in moisture is not only in influencing the texture, but also the composition of the cheese. The difference in moisture content between Cheddar and Camembert cheese is approximately 25 per cent. If it is supposed that the moisture of the cheese will have about the same sugar content as milk, this means that the sugar content of the Camembert cheese will be about 1 per cent greater than Cheddar; and since the sugar is all fermented, differences in sugar content ultimately result in differences in acidity.

One of the basal differences between Cheddar and Swiss cheese is that during the making of the first—that is, the operations before pressing—a large amount of acid is formed in the curd, while in the case of Swiss no such acid production occurs. Van Slyke and Hart,[1] Boekhout and Ott de Vries,[2] and Van Dam[3] have shown that acid is held chemically by the proteins of cheese, while sugar is not. Thus if in the making of one cheese acid is formed, and in another not formed, the result will be a somewhat different composition, even though the moisture content of the two cheeses is initially the same.

[1] Van Slyke, L. L., and Hart, E. B. A study of some of the salts formed by casein and paracasein with acids: Their relations to American Cheddar cheese. New York Agricultural Experiment Station Bulletin 214. Geneva, July, 1902. See p. 60.

[2] Boekhout, F. W. J., and Ott de Vries, J. J. Über den Käsefehler "Kurz" (Kort). Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 24, No. 5/7, pp. 122–129. Jena, Aug. 2, 1909.

[3] Van Dam, W. Über die Konsistenz der Käsemasse bei Edamkäsen. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 32, No. 1/2, pp. 7–40. Jena, Dec. 5, 1911.

Another difference in composition of cheese is caused by variations in the amount of salt and in the time at which it is added. This difference is an important one in the case of Cheddar and Swiss cheese. These differences in the initial composition are sufficient to exert a great influence on the types of life that are to grow in and on the cheese.

THE ENZYM CONTENT OF CHEESE.

The rôle of the inherent enzyms of milk and those of the rennet extract in the ripening of cheese have been emphasized by the work of Babcock and Russell and of Von Freudenreich and Jensen. There remains no doubt concerning the importance of certain enzyms, especially those of rennet extract, in the ripening of cheese. The enzymic content of all cheese must be much the same qualitatively, since they are made from the same raw materials. Again, it is difficult to see how one enzym can be active in one cheese and not in other kinds, since the conditions are quite similar as regards reaction and other factors. Accepting the theory of the specificity of enzyms, it must be admitted that the products of the activity of any enzym will be the same in kind no matter what the conditions under which it may be working. Hence it does not seem that the enzyms of milk or rennet can be factors determining the kind of cheese to be made by any method from the ordinary raw materials, but that the determining factors must be biological.

BIOLOGICAL PROBLEMS.

The methods of manufacture of cheese result in an environment that causes certain of the organisms constantly present in milk to develop in a definite sequence. The empirical methods of the cheesemaker result in a cheese that approximates, if it is not identical with, the normal of its kind.

Each of the great commercial varieties of cheese thus presents a distinct problem for the bacteriologist, who should be able to demonstrate the constant presence of the essential groups of bacteria. It can not be expected here any more than in most decomposition processes that a single specific organism will be concerned, as in the causation of disease, but rather that groups of organisms will be present, the basis of grouping being largely biochemical. Neither can it be expected that one group will grow for a period, then disappear and be followed by a second, but rather that the sequence of development will be confused.

The work of Thom on Camembert cheese furnishes an excellent illustration. It has been shown that a definite balance between the various forms of life concerned is essential for the normal ripening

of this cheese. If through methods of manufacture or through conditions of ripening this balance is destroyed, the cheese will not be typical.

The biological factors concerned in the ripening of Camembert cheese are the acid-forming bacteria, the Camembert mold (*Penicillium camemberti*), or its white form (*Penicillium camemberti* var. *rogeri*); *Oidium lactis;* and bacteria which together with *Oidium lactis* form a reddish slime on the surface of the cheese. If conditions favor the growth of *Oidium* lactis at the expense of the penicillium, the cheese will not be normal; if the mold is too luxuriant, texture is obtained but flavor is not. It is thus evident in the case of Camembert cheese too wide variations in the balance between the different forms of life can not occur if a cheese normal in texture and flavor is to be obtained. A large amount of work has been done on Camembert cheese and the necessity for the presence of the different forms established, but their exact rôle has not been shown and never can be until the chemist devises means of following in detail the complex chemical changes.

It is not only necessary that the constant presence of any organism in cheese be demonstrated in order to prove its importance in the ripening, but it must also be present in sufficient numbers so that it is certain that growth has taken place in the cheese. The simple presence of any form is no evidence of its activity. Such an error was made by Duclaux, one of the first bacteriologists to occupy himself with cheese problems. Since there are such a multitude of forms of bacteria in milk, and hence in cheese, any particular method of examination of the cheese is likely to bring some one group of organisms to the front. The methods used by Duclaux in his examination of Cantal cheese were fitted to favor the development of the spore-bearing, liquefying bacteria. Their constant presence, together with the fact that they produced in pure culture in milk compounds which he had already demonstrated in the ripe cheese, led him to consider them an important factor in the ripening of this cheese. It has been demonstrated since that the conditions in hard cheese do not permit this class of bacteria to develop.

BACTERIOLOGICAL METHODS.

The modern bacteriological methods are not such as will demonstrate the presence of all kinds of bacteria in milk, cheese, or any other substance in which a considerable number of kinds of bacteria are present, even though all may find conditions that will permit of growth on the medium employed. It is usually easy to demonstrate the presence of the form that occurs in greatest numbers, since in the more lightly inoculated culture plates this form will be present

in pure culture or else so freed from competition with the other forms that its growth will not be prevented. For those forms present in less numbers this condition does not obtain, and usually special methods must be employed to demonstrate their presence, such as the use of differentiating media or enrichment cultures. Anyone who has much experience in plating milk on lactose media has noted that in certain cases the plates thickly seeded show colonies of but a single organism, usually the ordinary lactic organism *Bacterium lactis acidi*, while on the more thinly seeded plates from the same sample other forms may appear. In the thickly seeded plates the lactic organisms, on account of favorable conditions, have grown most rapidly and by the acid produced have prevented the development of other forms, while in the more thinly seeded plates the colonies may be separated by such a distance that the products of one colony do not reach the others, each colony then grows as though it were the only colony on the plate. Hence forms appear on thinly seeded plates that do not on those crowded with colonies. If the organism that finds most favorable conditions greatly exceeds in numbers some other form the latter may never appear on the culture or be so inconstant as not to attract the attention of the bacteriologist. A ratio of 1,000 to 1 or even 500 to 1 may prevent the detection, by use of the ordinary plate-culture methods, of the organisms present in smaller numbers. This statement may serve to explain the results obtained by previous investigators of Cheddar cheese.

Before 1896 no consecutive bacteriological examinations had been made of a ripening Cheddar cheese. The first detailed work on this variety of cheese with which the writers are acquainted is that of Russell.[1] This work was largely concerned with a quantitative examination of the cheese during the ripening process by means of gelatin plates. It established the fact that acid-forming organisms make up 99 per cent and over of the bacteria thus determined. It may be inferred that the acid-forming bacteria belonged to the *Bacterium lactis acidi* group. The work of Lloyd on English Cheddar cheese led to similar results. The most extensive work on the bacterial flora of Cheddar cheese is that of Harding and Prucha,[2] who isolated the different types of organisms appearing on cultures made from 9 normal Cheddar cheeses during the entire period of ripening. The more than 300 cultures thus isolated were studied in detail and reduced to 33 groups. Of these 33 groups 4 belonged to the *Bacterium lactis acidi* group, the only one which was always found, and it practically always included over 99 per cent of the total germ

[1] Russell, H. L. The rise and fall of bacteria in Cheddar cheese. Wisconsin Agricultural Experiment Station, Thirteenth Annual Report, pp. 95–111. Madison, 1896.

[2] Harding, H. A., and Prucha, M. J. The bacterial flora of Cheddar cheese. New York Agricultural Experiment Station, Technical Bulletin 8. Geneva, Dec., 1908.

content. The 10 other groups which these investigators classed as important on account of frequency of occurrence were not found in all of the cheeses, and it may be inferred that they do not represent essential factors in the ripening of Cheddar cheese. In speaking of one cheese the authors say:

> The results from this cheese accord with the idea that aside from the lactic group there is no single group or at least no single species of bacteria absolutely essential to the ripening process.

The work of previous investigators has shown that the liquefying, the gas-forming, and the inert bacteria are not essential factors in the ripening of Cheddar cheese, since all or any one of these groups may be absent and yet the cheese may ripen in a normal manner. It is true that all of these groups are usually represented in Cheddar cheese, since they are present in milk, but the numbers are small, and in the case of the liquefying organisms there is no evidence that growth ever occurs during the ripening process.

Yeasts can not be classed as an important factor since they may be absent from a normal cheese.

The work of previous investigators may be summarized in the statement that the group of bacteria represented by *Bacterium lactis acidi* is the only one that up to the present has been shown to be of constant occurrence in Cheddar cheese. This fact together with the enormous numbers, amounting to millions and at times over a billion per gram of the fresh cheese, leaves no doubt of the importance of this group of organisms in Cheddar cheese, and undoubtedly in all cheese that undergoes a ripening process.

THE ROLE OF BACTERIUM LACTIS ACIDI IN CHEDDAR CHEESE.

The empirical methods of the Cheddar cheesemaker demand a milk so far advanced in the acid fermentation that during the few hours between time of curdling and placing the curd in the press acid development is rapid. The increasing acidity, as has long been known, favors the curdling of the milk by the rennet. Indeed the tests most frequently used in cheesemaking to determine the "ripeness" or acidity of the milk are those in which the time of curdling is noted, when a definite amount of rennet is added to milk at a definite temperature (tests of Monrad and Marschall). The Cheddar maker desires milk that has just passed through the "period of incubation." By this expression is meant the time during which no apparent increase in acidity results from the growth of acid-forming bacteria. This has been supposed to be due to the fact that no acid was formed by the rapidly growing organisms. More recently it has been pointed out by Rahn [1] that this is not the true explanation but that until the

[1] Rahn, Otto. The fermenting capacity of the average individual cell (*Bacterium lactis acidi*). Science, new series, vol. 33, No. 849, pp. 539–540. New York, Apr. 7, 1911.

bacteria have increased to a great extent the amount of acid formed is so small as to escape detection. Whatever the explanation, it is true that the acid-forming bacteria may increase until there are millions per cubic centimeter and yet the acidity show no change whatever, as has been shown by Koning[1] and by Burri and Kürsteiner.[2]

At last the acidity begins to develop with increasing rapidity until it reaches a point where it begins to exert an inhibitive effect upon the growth of the organism. In Table 1 are given some data illustrating the rate of acid development in milk kept at 95° F. It will be noted in both samples that the initial rate of increase in acid is small, that it reaches a maximum that may be over 0.15 per cent per hour and then declines. Both samples of milk were slightly too acid for cheesemaking.

TABLE 1.—*Rate of development of acid in milk kept at 95° F.*

	Hours.					
	0	1	2	3	5	24
Sample 1:	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Acidity	0.24	0.30	0.40	0.47	0.71	1.00
Increase per hour	.....	.06	.10	.07	.12	.015
Sample 2:						
Acidity	.20	.24	.34	.5	.74	.99
Increase per hour	.....	.04	.10	.16	.12	.012

This rapid increase in acidity is the result of the growth of the acid-forming bacteria in the favorable environment. In Table 2 are given the results of the bacteriological examination of two samples of milk kept at 95° F. for a number of hours. It will be noted that the increase in numbers of bacteria, like that of the acidity, goes on with increasing rapidity until a maximum is reached, after which the growth is less and less rapid.

TABLE 2.—*Increase of bacteria per cubic centimeter in milk kept at 95° F.*

[Number of bacteria expressed in millions.]

	Hours.					
	0	2	4	6	8	10
Sample 1	59	83	231	927	1,064	1,167
Sample 2	4	.....	157	826	1,545	1,982

[1] Koning, C. J. Der Säuregrad der Milch. [Pharmaceutisch Weekblad, 1904] Milchwirtshaftliches Zentralblatt, vol. 1, No. 7, pp. 289–305, July; No. 8, pp. 337–356, August. Leipzig, 1905. See p. 294.

[2] Burri, R., and Kürsteiner, J. Untersuchungen über die Reifung der Käsereimilch. Landwirtschaftliches Jahrbuch der Schweiz, vol. 24, pp. 437–466. Bern, 1910.

It is essential that the acid development should be rapid during the time between the curdling of the milk and pressing the curd. The modern Cheddar maker insures this by the addition of large numbers of acid-forming bacteria in the form of a starter.

EFFECT OF CURDLING ON DISTRIBUTION OF BACTERIA IN MILK.

The solid bodies present in the milk will be held by the curd in the same manner as the formation of aluminum or ferric hydrate in water enmeshes the solid bodies present, or the coagulating of albumen in a solution removes turbidity therefrom, as in the clearing of bacteriological media and of wine.

In order to illustrate the effect of curdling on the distribution of bacteria several samples of milk were subjected to a quantitative examination. Rennet solution was then added and the curd cut. As soon as possible a sample of the whey was likewise examined. In Table 3 are given the data obtained from a number of such examinations. It will be noted that in all cases a unit volume of the whey contained less bacteria than the milk before curdling. From the average figures of the determinations approximately 77 per cent of the bacteria were retained in the curd in the trials made in the laboratory.

TABLE 3.—*Number of bacteria per cubic centimeter in milk and in whey immediately after curdling—Laboratory experiments.*

[Number of bacteria expressed in thousands.]

	Trial No.					
	1	2	3	4	5	6
Milk	128,000	11,000	2,050	155,000	23,000	59,000
Whey	34,000	2,900	730	756	773	776

A number of similar examinations were made under practical conditions in the cheese room, the sample of milk being taken from the cheese vat and the whey after cutting the curd in the usual manner. The data are given in Table 4. It will be noted that the results are similar to those obtained in the laboratory. Approximately 73 per cent of the bacteria in the milk were retained in the curd.

TABLE 4.—*Number of bacteria per cubic centimeter in milk and in whey immediately after curdling—Samples from the cheese vat.*

[Number of bacteria expressed in thousands.]

	Trial No.				
	1	2	3	4	5
Milk	5,600	6,400	5,430	4,000	6,500
Whey	3,000	1,290	2,400	210	460

The result of curdling and the shrinking of the curd is that the acid-forming bacteria of the milk are concentrated in the curd, which soon after cutting occupies but a fraction of the volume of the original milk. This concentration, together with the favorable environment, results in a rapid formation of acid in the curd.

This condition should result in a more rapid increase of acid in a whey in which the curd is allowed to remain than in a portion of the same whey removed from the curd. As the curd shrinks the expelled whey should bring with it a portion of the acid that has been formed in the curd; this, together with osmotic action, should increase the acidity of the whey in contact with the curd.

In order to demonstrate this a sample of milk was curdled, the curd cut, and as soon as possible a portion of the whey removed to a separate vessel. Acidity determinations were made at intervals. The results are given in Table 5. It will be noted that in every case the acidity of the whey in contact with the curd increased much more rapidly than that of the whey not in contact with the curd.

TABLE 5.—*Development of acid in whey alone and in whey in contact with curd.*

	Hours.						
	0	2	4	6	8	10	12
Sample 1:	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Whey	0.09	0.09	0.10	0.13	0.16	0.24	0.36
Whey and curd	.09	.10	.16	.17	.32	.47	.54
Sample 2:							
Whey	.14	.20	.30	.35	.49	.....	.....
Whey and curd	.14	.27	.48	.53	.68	.....	.....
Sample 3:							
Whey	.10	.12	.12	.22	.33	.....	.....
Whey and curd	.10	.12	.16	.32	.49	.....	.....
Sample 4:							
Whey	.09	.10	.11	.13	.14	.33	.....
Whey and curd	.09	.10	.13	.18	.20	.42	.....

It was thought that if a sample of milk was curdled by rennet and the curd cut and allowed to settle to the bottom of a deep container, the whey at different levels should show varying degrees of acidity if the container were so protected that convection currents did not tend to mix the liquid. In other words, the acid whey expelled would tend to accumulate in the lower portion of the container.

In order to test this, deep beakers were filled with milk, the milk curdled with rennet, and the curd cut and allowed to settle. The beakers were kept in a thermostat at 95° F.; thus no convection currents were present, since care was taken to heat the milk to the same temperature before curdling. The samples of whey were removed in such a manner as not to mix the different layers. In Table 6 the results of a number of trials are given. It will be seen that the acidity of the bottom layers increases much faster than that of the upper layers, the difference being in some cases 0.4 per cent.

TABLE 6.—*Acidity of whey at the top and bottom of a vessel containing whey and curd.*

	Hours.						
	0	2	4	6	8	10	12
Sample 1:	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Top	0.09	0.09	0.11	0.12	0.16	0.27	0.40
Bottom	.09	.10	.13	.22	.49	.67	.68
Sample 2:							
Top	.14	.21	.32	.36	.56	.....	.....
Bottom	.14	.34	.64	.71	.80	.....	.....
Sample 3:							
Top	.08	.08	.09	.09	.12	.18	.24
Bottom	.08	.08	.09	.11	.15	.34	.45
Sample 4:							
Top	.09	.10	.13	.14	.17	.25	.....
Bottom	.09	.10	.14	.22	.24	.59	.....

It is thus shown in a number of ways that the location of acid development is in the curd rather than in the whey.

It has been shown that paracasein will absorb and probably combine with acids. If this is so, a portion of the acid formed in the curd should be retained in loose chemical combination and the acidity of a sample of milk should increase more rapidly than that of the whey from the same milk, even though the whey is in contact with the curd. The data given in Table 7 show this to be true. The figures represent the increase in acid at the end of each period over the initial acidity of the milk or whey and curd. This leaves no doubt that a portion of the acid is retained by the curd, and is proof of an earlier statement, that a cheese in which acid is developed during the process of making will have a different acidity from one with an equal content of moisture, but in which no acid is formed during the making.

TABLE 7.—*Increase in acidity of milk and of whey in contact with curd.*

	Hours.					
	2	4	6	8	10	12
Sample 1:	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Milk	0.19	0.34	0.64	0.73	.....	.....
Whey and curd	.13	.32	.38	.52	.....	.....
Sample 2:						
Milk	.00	.01	.09	.32	0.55	0.74
Whey and curd	.00	.01	.02	.05	.18	.26
Sample 3:						
Milk	.04	.21	.44	.69	.....	.....
Whey and curd	.05	.11	.16	.28	.....	.....
Sample 4:						
Milk	.03	.15	.43	.63	.....	.....
Whey and curd	.03	.04	.16	.25	.....	.....

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EFFECT OF ACID ON EXPULSION OF THE WHEY.

It has been shown by Sammis, Suzuki, and Laabs [1] that the expulsion of whey from the curd is directly proportional to the amount of acidity present; at least, in the case of percentages of acidity that are met with in normal cheese curds. The growth of lactic-acid bacteria in the curd and the consequent development of acidity are necessary to secure a curd with the proper moisture content.

EFFECT OF ACID ON THE TEXTURE OF THE CURD.

The curd from milk in which no growth of acid-forming bacteria has taken place shows but a slight tendency to mat or for the pieces of curd to fuse. If, however, acid is formed in the pieces of curd, they undergo such a change in texture that as soon as they are allowed to settle to the bottom of the vat they soon unite to form a single mass of curd. In the making of cheese by the Cheddar process it is essential that this matting take place.

The change in texture which the curd undergoes is due to the action of the acid on the paracasein, forming a substance that, when warmed, can be drawn into threads. The basis of the "hot iron" test as used to determine the time to draw the whey is the formation of this compound by the acid.

At the time the curd is placed in the press there are numerous spaces between the curd particles. It is essential that the curd be so plastic that, under the influence of the pressure, the particles undergo perfect fusion, so that the entire cheese is one mass, perfectly free from irregular-shaped mechanical holes. Such a fusion does not occur in the absence of acid formation in the curd.

THE RÔLE OF ACID IN THE RIPENING OF CHEESE.

In the ripening process proper the acid resulting from the fermentation of the sugar by the organisms of the *Bacterium lactis acidi* group has an important rôle. As has been shown by numerous investigators, the sugar in the cheese is all fermented within a few days. It was shown by Babcock and Russell,[2] also by Jensen,[3] and by Van Slyke, Harding, and Hart [4] that rennet extract not only has a curdling effect but has a digestive action in the presence of an

[1] Sammis, J. L.; Suzuki, S. K., and Laabs, F. W. Factors controlling the moisture content of cheese curds. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 122. Washington, 1910.

[2] Babcock, S. M.; Russell, H. L.; and Vivian, A. Influence of rennet on cheese ripening. Wisconsin Agricultural Experiment Station, Seventeenth Annual Report, pp. 102–122. Madison, 1900.

[3] Jensen, Orla. Studien über die Enzyme im Käse. Landwirtschaftliches Jahrbuch der Schweiz, vol. 14, pp. 197–233. Berne, 1900.

[4] Van Slyke, L. L.; Harding, H. A.; and Hart, E. B. Rennet-enzyme as a factor in cheese ripening. New York Agricultural Experiment Station, Bulletin 233. Geneva, June, 1903.

activating acid such as is present in the cheese. The rapid proteolysis occurring during the first part of the ripening process is largely due to the action of the pepsin.

Variations in the amount of rennet extract added to the milk result in differences in the rate of ripening, as has been shown by numerous investigators. This can be explained only through the variations in the pepsin content of the cheese. The action of rennet extract in the presence and absence of lactic organisms is demonstrated in Plate I, figure 1. A 4 per cent solution of agar was added to sterile milk in the proportion of 1 to 1. A portion of the milk agar was heavily inoculated with a lactic organism and the plates incubated for 24 hours; the other portion was not inoculated. At the end of the period of incubation strips of filter paper were moistened with rennet extract and placed on the plates, which were then incubated at 37° C. for one hour. The photograph was taken at the end of the period. It will be noted that in the presence of the lactic organisms the casein has been rendered soluble by the rennet extract, while in the absence of the organisms no such marked digestion has occurred. The digestion of the casein has resulted in the destruction of the opacity of the milk agar so that a number placed beneath the dish can be read, while in the case of the milk agar from which the lactic organisms were absent no trace of a similar number can be distinguished. In the milk agar conditions are very similar to those obtaining in cheese, the activating acid being of like origin.

It would thus seem that if a cheese was made from milk that contained but few acid-forming organisms the rate of ripening would be delayed. In order to test this hypothesis, a cheese was made from very clean milk, containing scarcely any acid-producing organisms, and no starter was added. No acid whatever was formed during the making process. The milk after standing 24 hours at 20° C. had an acidity of but 0.19 per cent. In order to insure proper curdling, the acidity of the milk was raised to 0.25 per cent by the addition of hydrochloric acid. In order to follow the rate of acid formation, determinations of the sugar present in the cheese were made at intervals. The results are given in Table 8.

TABLE 8.—*Sugar content of cheese made from milk containing few lactic bacteria.*

Time.	Sugar content.	Acidity.	Time.	Sugar content.	Acidity.
Days.	*Per cent.*	*Per cent.*	*Days.*	*Per cent.*	*Per cent.*
1	1.51	.25	8	1.45	.27
2	1.49	.27	22	.94	.45
4	1.48	.27	46	.00	1.13

The sugar disappears from a normal cheese in 3 to 5 days, while on the twenty-second day over one-half of the sugar remained in the experimental cheese. The maximum bacterial content, as measured by the ordinary plate cultures, was not attained until after the sixth week. The rate of ripening, as measured by the change in texture and development of flavor, was correspondingly slow. At three months the cheese still showed a spongy texture and scarcely any cheese flavor.

THE PROTECTIVE ACTION OF ACID.

The putrefactive bacteria of the groups that are constantly present in milk, and hence in cheese, are unable to grow on account of the acid reaction which is maintained during the entire period of ripening. This protective action of acid was first demonstrated at the Wisconsin Experiment Station by Babcock and Russell,[1] who removed the sugar from curd by washing in water. The cheese developed most undesirable odors and tastes, and bacteriological examinations showed liquefying bacteria to be numerous. If lactose, glucose, or cane sugar were added to the washed curd, the ripening process was much more nearly normal because the acid reaction was thereby restored, due to the fermentation of the added sugar, and the putrefactive organisms were inhibited as in a normal cheese. In the experimental work on flavor development in Cheddar cheese, a number of cheeses were prepared from curd from which the sugar was removed by washing. In Plate I, figure 2, a normal and a washed-curd cheese are illustrated. The washed-curd cheese was devoid of texture, being a soft, plastic mass and having no resemblance to cheese in odor and taste. On the right of the picture is a cheese made from the same washed curd to which acid had been added, with the result that the firmness was restored.

The rôle of organisms of the *Bacterium lactis acidi* group in Cheddar cheese may be summarized as follows:

1. They favor the curdling process.
2. They favor the expulsion of the whey.
3. They permit of the fusing of the curd particles.
4. They activate the pepsin of the rennet extract.
5. They have a protective action against the putrefactive bacteria.

It is certain that this group is an essential factor in the ripening of Cheddar cheese. Their period of growth has been believed to be short, since it has not been supposed they can continue to develop after the disappearance of the sugar. It has been shown by numer-

[1] Babcock, S. M., Russell, H. L., Vivian, A., and Hastings, E. G. Influence of sugar on the nature of the fermentations occurring in milk and cheese. Wisconsin Agricultural Experiment Station, Eighteenth Annual Report, pp. 162–176. Madison, 1901.

Fig. 1.—The Action of Rennet Extract on Casein Suspended in Agar in the Presence and in the Absence of Acid-Forming Bacteria.

The same number (902) was placed beneath both plates at the time the photograph was taken. The digestion of the casein in the presence of the acid-forming bacteria has rendered the medium transparent and the number apparent, while in the absence of the acid-forming bacteria the digestive action has been almost nil.

Fig. 2.—The Effect of Acid on Cheese.

On the left is a cheese made from normal milk by the usual process; in the center a cheese made from the same milk, the curd being washed free of sugar; on the right a cheese made from the same washed curd, to which acid had been added. The washed-curd cheese had no body and was a soft, plastic mass. The addition of acid restored the firmness of the body.

ous investigators that the period of maximum numbers is when the cheese is a few days old; that thereafter the decline in numbers is more or less rapid. In some cheeses, as will be shown later, high numbers may persist for long periods of time. It is not usually believed that they can exert any marked action in other lines than those that have been mentioned after growth ceases, although such a belief may not be well founded. Indeed, some facts would indicate otherwise.

The ripening process, both in regard to proteolysis and development of flavor, continues long after what is supposed to be the period of bacterial activity. A large amount of data is available as to the proteolytic changes going on in the ripening cheese, but until recently no data have been available to show that the compounds containing no nitrogen were likewise constantly changing. The content of the cheese in volatile fatty acids is constantly changing, and especially the relative amounts of the different acids. To explain these changes recourse must be had to enzyms, and most probably to those elaborated by the lactic organisms or to other types of bacteria that develop subsequently to what has been called the period of bacterial activity.

The data to be presented will show that the period of activity is not to be measured by determining the ordinary types of bacteria. The demonstration of continued bacterial growth does not, of course, eliminate the action of enzyms formed by the *Bacterium lactic acidi* group of organisms that during the early life of the cheese make up most of the flora.

THE RATE OF BACTERIAL GROWTH IN CHEESE.

In the course of the cooperative work carried on by the Dairy Division of the Department of Agriculture and the Wisconsin Experiment Station a large number of cheeses have been examined bacteriologically. Some were examined at frequent intervals during the making process and during the first portion of the ripening period; others were examined at less frequent intervals during the entire period of ripening.

METHODS OF EXAMINATION.

The method of examination used was the grinding with sterile quartz sand of a sample of the cheese taken from various parts of a plug and handled under aseptic conditions. Inoculations in varying dilutions were made from the cheese suspension into lactose-agar plates. At first the period of incubation of the plates was 48 hours at 37° C. Later the incubation at 37° was supplemented by a further incubation at 20°. This method of incubation allows the develop-

ment of the maximum number of organisms and insures the growth of the colonies to a maximum size, an important point when it is desired to differentiate the various types present. Gelatin containing no added sugar was used in a portion of the work; the plate cultures were then incubated at 18° to 22° C. for 10 days and for even longer periods when slightly lower temperatures were employed.

RESULTS OF THE WORK.

The data collected by means of the methods that have been used by other investigators of Cheddar cheese serve simply to confirm their work and to emphasize the number of organisms of the *Bacterium lactis acidi* group that are to be found during the early part of the ripening period. A portion of the data is presented in Tables 9, 10, and 11 because it gives a very complete picture of the bacterial development during and subsequent to the making of the cheese.

The maximum number of bacteria as measured by the lactose-agar plate cultures occurs early in the ripening process. In 8 of the 11 cheeses examined, as shown in Tables 9 and 10, the greatest number of bacteria was found within 48 hours. In the case of the examinations detailed in Table 11, it will be noted that the maximum numbers of bacteria as determined by lactose-agar plates have been found much later, namely, at the end of the fourteenth, forty-fifth, and seventy-seventh days. Thus, in no case was the maximum number found at the first determination. It is not believed, however, that these cheeses, selected especially to illustrate certain points to be discussed later, give a true picture of the initial development of bacteria. In the case of 9 of the 13 to be mentioned later, the maximum number of bacteria was found on the first, second, or third examinations. Harding and Prucha [1] obtained similar results. Seven out of 9 cheeses examined showed the maximum number of bacteria at the first examination, 1 at the second, and 1 at the fourth.

It is impossible to determine the time at which the growth of any particular type of organism in cheese ceases. As the fermentation in the cheese progresses and the accumulation of by-products increases, cell death begins to occur. As long as the process of cell division is more rapid than death of the cells, an increase in living bacteria will be shown by the plate cultures. Soon death of the cells is more rapid than cell division. At this point the decrease in apparent numbers begins, although growth may continue for a much longer period. It seems probable that the growth of the bacteria of the *Bacterium lactis acidi* group continues until the sugar is completely fermented. In milk the cessation of growth is due to the

[1] Harding, H. A., and Prucha, M. J. The bacterial flora of Cheddar cheese. New York Agricultural Experiment Station, Technical Bulletin 8. Geneva, Dec., 1908.

appearance of free acid, in cheese to the disappearance of an essential food, a fermentable carbohydrate.

The large numbers of bacteria found during the first days are striking; the number as given in the tables is far below the actual number, due to the impossibility of breaking up the colonies in the tough cheese. It is probable that the germ content of cheese often amounts to hundreds of billions of living bacteria in each gram of the moist cheese. From what is known of the number of cells in a gram of moist bacterial growth, it is certain that at the time the maximum numbers of the organisms of the *Bacterium lactis acidi* group are found, at least 0.1 per cent of the moist cheese consists of bacteria.

TABLE 9.—*Numbers of bacteria per gram in Cheddar cheese as determined by lactose-agar plate cultures.*

[Numbers expressed in millions.]

Cheese No.	Days.										
	0	1	2	3	4	5	6	7	9	10	12
434	31	.....	.....	490	.....	537	489	21	.....	43	.....
438	4.5	.....	174	.....	134	292	3	.....	.....	1	.....
442	1.3	971	831	133	.....	.....	.....	221	.....	.....	.....
474	58	1,657	987	.....	325	.....	407	.....	239	.....	141
475	158	1,538	1,344	987	.....	.....	545	.....	69	.....	736
476	138	1,311	2,518	.....	616	.....	195	.....	293	.....	728
477	53	1,925	2,171	.....	1,916	.....	221	.....	65	.....	598

TABLE 10.—*Numbers of bacteria per gram in Cheddar cheese as determined by lactose-agar plate cultures.*

[Numbers expressed in millions.]

Cheese No.	Milk.	Curd at salting time.	12 hours.	Days.										
				1	2	4	6	14	21	28	35	49	70	98
580	8	160	332	586	235	145	165	51	284	285	104	132	128	114
581	0.5	326	1,048	736	405	684	184	211	290	453	261	228	291	212
582	.7	912	623	709	848	522	853	369	348	314	326	436	193	45
583	.5	839	965	569	580	1,025	184	401	319	144	504	661	168	55

TABLE 11.—*Numbers of bacteria per gram in Cheddar cheese as determined by plate cultures.*

[Numbers expressed in millions.]

Number of days.	Cheese No. 1.		Cheese No. 54.		Cheese No. 91.		Cheese No. 92.	
	Lactose-agar culture.	Gelatin culture.	Lactose-agar culture.	Gelatin culture.	Lactose-agar culture.	Gelatin culture.	Lactose-agar culture.	Gelatin culture.
2					150		250	150
4	3							
8					61	120	400	340
11	8	14						
14			32		1,400	530	500	
22	2.5	10			58	1,600	35	
30	2	10	15	70				
37					270	480		100
45	40	25			1,400		340	
56			32	51	1,300	250	225	225
77			51	40				
86	36.5	24			128	320	250	84
100			8.5	12	74	65	100	120
113	5	5			145	142	132	87
124			14	12	50	28.5	145	96
143	13	13	22.5	12.5	18	23.5	38.5	38
155					15	10.5		32
165					17.5	5.5	63	
176	3.5	2.5					9	5.5
187			6		7.5		12.5	2

According to the determinations of MacNeal, Latzer, and Kerr,[1] there are about 5,300 billion colon bacilli in a gram of dry growth. Figuring on a moisture content of 90 per cent, there would be 530 billion in a gram of the moist growth. From determinations made by weighing out 1 gram of the moist growth of a coccus form from cheese, making a uniform suspension of this in a known volume of water, and taking an equal volume of this suspension and of normal blood and counting the number of bacteria and red corpuscles we have obtained an average figure of 1,150 billion cells per gram of the moist growth. The basis for this method of counting is the fact that from a known number of red blood cells one can figure the volume used, since the red cells are practically a constant quantity. The average volume of the colon organism is 1.13 cubic microns; of the coccus 0.5236. The agreement in the determinations is thus very close.

It is not to be supposed that, so far as the number of bacteria is concerned, all cheese will be similar. The results shown in Table 11 emphasize this point; two of the cheeses, Nos. 1 and 54, showed at no time over a few million bacteria per gram, while the remaining two had a consistently high germ content. Such differences may be due to many causes. The period of maximum numbers is followed by a decline, rapid in some instances, slow in others. At the time the cheese is fully ripe millions of living lactic bacteria are usually pres-

[1] MacNeal, Ward J.; Latzer, Lenore L.; and Kerr, Josephine E. The fecal bacteria of healthy men. Journal of Infectious Diseases, vol. 6, No. 2, pp. 123–169, Apr. 1; No. 5, pp. 571–609, Nov. 26. Chicago, 1909.

ent in each gram of cheese. Living lactic organisms have been found by one of the authors in a cheese over 4 years old.

The ripening of the cheese, both in reference to proteolysis and flavor development, continues long after this group of organisms has ceased to grow. A large amount of data is available to show that a constant change in the nitrogenous bodies present is taking place. It has also been shown by the authors that the content of the cheese in fixed and volatile acids changes during the ripening process. To explain these latter changes, recourse must be had to enzyms elaborated by the lactic bacteria, or else it must be supposed that other groups of organisms develop subsequently.

THE ENZYMIC ACTION OF LACTIC BACTERIA.

The work of Buchner, Herzog, and others has shown the presence of an enzym in certain lactic-acid-producing bacteria that are essentially different from those predominating in cheese. This intracellular enzym, which can be demonstrated only after the disintegration of the cell, forms small quantities of lactic acid from sugar. So far as is known to us, a similar enzym has never been demonstrated in organisms of the *Bacterium lactis acidi* group. The growth of these organisms on all media is so meager that it is very difficult to obtain a sufficient amount of the growth so that it can be treated by methods similar to those employed by Buchner and others. An acid-producing enzym in the lactic bacteria has, however, been demonstrated by quite different methods. It had been noted that when a sample of raw or sterilized milk in which varying numbers of lactic bacteria had been allowed to develop, and to which a preservative, as chloroform or toluol, had been added, the cells soon disappeared, or at least could no longer be detected by microscopical examination. It was thought that if the cells would undergo disintegration after having been killed by an antiseptic while in an actively growing condition, any enzyms present should exert their peculiar action as well as though the cells were mechanically ruptured.

The following experiment was planned: Bottles of fresh raw milk and of the same milk heated to 97° C. for a short time were inoculated with a pure culture of *Bacterium lactis acidi*. At varying periods in the development of acid a portion of the milk was removed and preserved with 3 per cent of toluol. In the bottles treated soon after inoculation a small number of bacteria were present, while in those to which the preservative was added at a later stage in the development of acid a much larger amount of bacterial growth was present. If any enzymic action occurred, the bottles should show a quantitative difference in the amount of acid formed corresponding to the amount of bacterial cells present. Raw milk, when preserved

with chloroform or toluol, gradually increases in acidity. This increase occurs when the milk contains only the bacteria that come from the interior of the udder and to which the preservative has been added as soon as drawn. In a bottle of milk put up in 1898 by Dr. S. M. Babcock and preserved with an excess of chloroform an acidity of 0.7 per cent was found in 1910, while the sugar content was as great as in the fresh milk, being 5 per cent. This increase of acid has been shown in all samples of milk preserved by the authors. It is undoubtedly due to the formation of amino acids by the inherent proteolytic enzyms of the milk.

In the bottles of raw milk in the experiment two acid-forming factors might be present: First, one forming an acid from the protein; second, the enzym of the lactic bacteria acting on the sugar. In the bottles of heated milk only the latter could be active, since the degree of heat was sufficient to destroy all the inherent enzyms of the milk. The milks thus differently treated should show a quantitative difference in increase in acid if the bacterial enzyms were capable of such action. The first bottle of the raw-milk series contained a minimum number of bacteria, since the milk was but a few hours old and drawn under clean conditions. The acidity in this bottle was that of the fresh milk. Three per cent of a pure lactic organism in milk was then added to the remainder of the raw milk. The second bottle was filled immediately after the addition of the culture. The milk was then incubated and at varying intervals bottles were filled and toluol added. The first acid determinations were made at once after adding the toluol to each bottle. It will be noted in Table 13 that the acidity in the case of the raw milk varied from 0.184 to 0.408 per cent. The increasing acidity indicated a great increase in the numbers of bacteria, which was also shown by microscopical preparations made from each bottle at the time the preservative was added.

The set of bottles filled with heated milk were treated in the same manner as the raw milk. The first bottle of this set contained practically no living bacteria. The acidity in the case of the remainder ranged from 0.203 to 0.456 per cent.

In order to determine how long the cells persisted in an active condition, tubes of milk were heavily inoculated from the various bottles. The results are given in Table 12.

TABLE 12.—*Results of inoculations of sterile milk from the bottles of milk containing 3 per cent toluol.*

Bottle No.	Tube.	Growth from inoculations made after—		
		1 day.	3 days.	5 days.
1.....	1	−	−	.....
	2	−	−	.....
2.....	1	−	−	.....
	2	−	−	.....
3.....	1	−	−	.....
	2	−	−	.....
4.....	1	−	−	.....
	2	−	−	.....
5.....	1	−	−	.....
	2	−	−	.....
6.....	1	−	−	.....
	2	−	−	.....
7.....	1	+	−	.....
	2	−	−	.....
8.....	1	−	−	−
	2	−	−	−
9.....	1	+	+	−
	2	−	+	−
10.....	1	−	−	−
	2	−	−	−
11.....	1	−	−	−
	2	−	−	−
12.....	1	+	−	−
	2	+	−	−
13.....	1	+	−	−
	2	+	−	−
14.....	1	−	+	−
	2	−	−	−

The results of the inoculation of sterile milk show that no growth could have taken place in the bottles after the addition of the toluol, and that within a short time the lactic organisms were all destroyed. Any acid formed in the raw milk after the fifth day must have been due in part, at least, to the inherent proteolytic enzyms of the milk; any increase of acid in the heated milk must have been due to the enzyms set free by the disintegrating cells.

Microscopical preparations were made at intervals from the various bottles. The smears were stained with a saturated aqueous solution of methylene blue. With this stain the cells can be distinguished for some time after they can no longer be demonstrated by the Gram-Weigert stain, which has been used in the examination of smears direct from cheese (See p. 32.) In raw milk the cells were agglutinated into large clumps. The number in the preparations made at various intervals became less and less, and after 45 days they had completely disappeared. As the cells deteriorated they stained more and more faintly.

In the heated milk the disappearance of the cells was less rapid. After 74 days some could be distinguished, although their outlines seemed to be more or less distorted.

In the bottles the following conditions were present: The mass of cells varied widely; cell growth undoubtedly ceased as soon as the antiseptic was added and within a few hours the cells were all destroyed, having been killed while in an active condition by an agent that is supposed to have the minimum effect on the intracellular

enzyms. The cells disintegrated more or less rapidly. It would seem that if any acid-forming enzyms were present in the bacterial cells they should manifest themselves under these conditions by the formation of acid. Acid determinations were made with the greatest precautions possible under the conditions of the experiment. The results are given in detail in Table 13:

TABLE 13.—*Increase of acidity in milk preserved with 3 per cent toluol.*

RAW MILK.

Sample.	When preservative was added.	Days.						
		1	13	28	59	90	121	150
Bottle No. 1:	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Acidity	0.184	0.19	0.234	0.273	0.29	0.317	0.368	0.368
Increase		.006	.05	.089	.106	.133	.184	.184
Bottle No. 2:								
Acidity	.204	.22	.257	.299	.326	.349	.414	.414
Increase		.016	.053	.095	.122	.145	.21	.21
Bottle No. 3:								
Acidity	.223	.261	.308	.377	.359	.4	.515	.506
Increase		.038	.085	.154	.136	.177	.292	.283
Bottle No. 4:								
Acidity	.266	.261	.326	.345	.391	.414	.492	.494
Increase		−.005	.06	.079	.125	.148	.226	.228
Bottle No. 5:								
Acidity	.282	.332	.404	.45	.492	.423	.644	.584
Increase		.05	.122	.168	.21	.141	.362	.302
Bottle No. 6:								
Acidity	.327	.356	.408	.443	.515	.538	.644	.676
Increase		.029	.081	.116	.188	.211	.317	.349
Bottle No. 7:								
Acidity	.408	.489	.561	.579	.616	.63	.745	.823
Increase		.081	.153	.171	.208	.222	.337	.415

HEATED MILK.

Bottle No. 8:								
Acidity	0.184	0.18	0.175	0.197	0.212	0.243	0.262	0.267
Increase		−.004	−.009	.013	.028	.059	.078	.083
Bottle No. 9:								
Acidity	.203	.206	.202	.225	.258	.27	.276	.276
Increase		.003	−.001	.022	.055	.067	.073	.073
Bottle No. 10:								
Acidity	.256	.285	.317	.331	.338	.354	.363	.372
Increase		.029	.061	.075	.082	.098	.107	.116
Bottle No. 11:								
Acidity	.308	.356	.368	.395	.394	.423	.432	.451
Increase		.048	.06	.087	.086	.115	.124	.143
Bottle No. 12:								
Acidity	.349	.427	.423	.46	.428	.492	.54	.543
Increase		.078	.074	.111	.079	.143	.191	.194
Bottle No. 13:								
Acidity	.41	.46	.45	.46	.497	.515	.561	.672
Increase		.005	.04	.05	.087	.105	.151	.262
Bottle No. 14:								
Acidity	.456	.494	.519	.552	.528	.599	.68	.708
Increase		.038	.063	.096	.072	.143	.224	.252

From the conditions of the experiment it is to be expected that the increase of acidity in the raw milk would be greater than in the heated milk, and that if the bacterial enzyms were operative the increase in acid would be directly proportional to the amount of enzym present or to the mass of cells. It will be noted that these are the conditions shown to be present by the figures given in the table. The data from three bottles of each of the raw and heated milks are given in graphical form in figures 1 and 2. The bottles

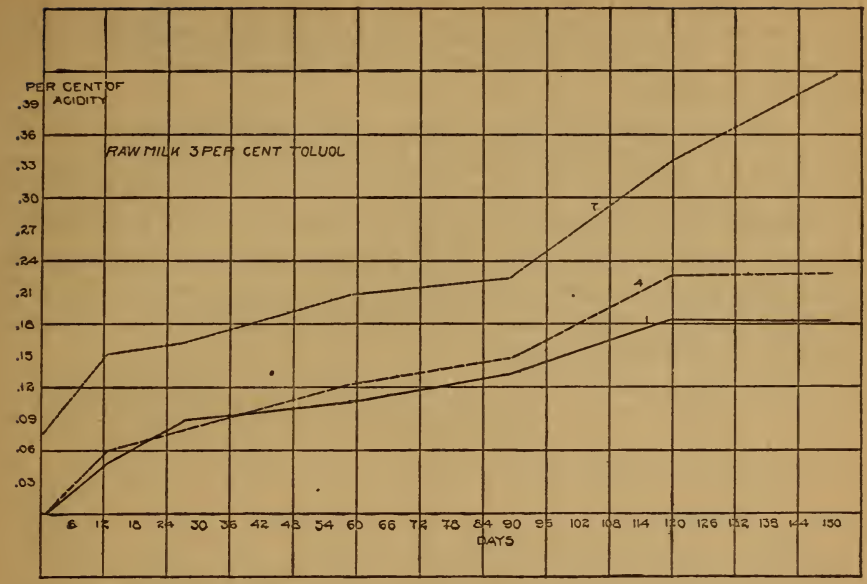

FIG. 1.—Increase of acidity in raw milk preserved with 3 per cent toluol. Bottle 1 contained the minimum number of bacteria, bottle 7 the maximum, and bottle 4 an intermediate number.

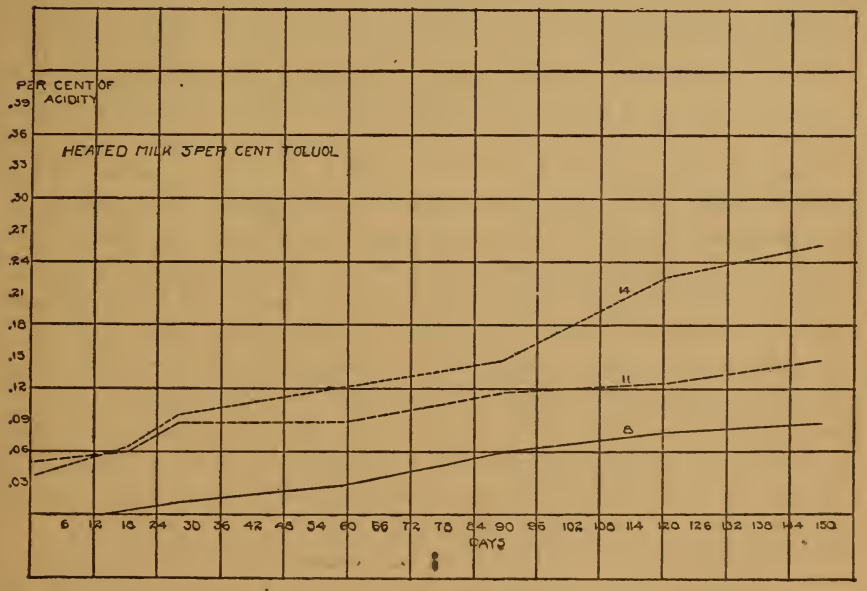

FIG. 2.—The increase of acidity in heated milk preserved with 3 per cent toluol. Bottle 8 contained the minimum number of bacteria, bottle 14 the maximum, and bottle 11 an intermediate number.

selected were the ones containing the minimum and maximum numbers of bacteria and an intermediate bottle from each set. The curves presented show the results to be entirely consistent with what might be expected.

In order to make the comparisons more easy there are given in Table 14 the rate of daily increase for each bottle of the two sets. The difference between the increase of acid in the raw milk and the heated milk should give the increase in the raw milk due to the inherent enzyms of the milk. This increase should be constant, or nearly so, since the same amount of enzym was operative in each bottle, although under somewhat different conditions, as regards acidity in the different bottles.

TABLE 14.—*Daily increase in acidity in bottles of raw and heated milk preserved with toluol.*

Raw milk.		Heated milk.		Increase (due to enzyms of milk).
	Per cent.		*Per cent.*	*Per cent.*
Bottle 1	0.0012	Bottle 8	0.0005	0.0007
Bottle 2	.0014	Bottle 9	.0005	.0009
Bottle 3	.0018	Bottle 10	.0007	.0011
Bottle 4	.0015	Bottle 11	.0010	.0005
Bottle 5	.0020	Bottle 12	.0013	.0000
Bottle 6	.0023	Bottle 13	.0017	.0007
Bottle 7	.0027	Bottle 14	.0017	.0016

It will be noted that the daily increase is proportional to the amount of bacteria present, and that the daily increase due to the inherent enzyms of milk is as constant as could be expected. The comparison has been made bottle for bottle in the two series. This introduces an error, since the amount of bacterial cells present in the compared bottles of raw and heated milk was not always the same. In the case of bottles 2 and 9, which are compared, the initial acidity was identical, while in the case of bottles 6 and 13, which are also compared, the initial difference in acidity was 0.083 per cent. The results seem to leave no doubt concerning the presence of an acid-forming enzym in the organisms of the *Bacterium lactis acidi* group that acts on the milk sugar. It might be thought that the increase in acidity was due to the production of amino acids by a proteolytic enzym. In this case the soluble nitrogen must be augmented. That this does not occur has been shown by numerous investigators.

The experiment was repeated in full detail with identical results. The increase in acid can not be asserted to be due to the formation of lactic acid, since, of course, no qualitative tests could be made in the presence of the lactic acid present in the milk at the beginning of the experiment.

It is true that in cheese the sugar disappears within a few days, and the enzym which was active under the conditions of the experiment could therefore not be active in cheese. This group of organisms has been believed to be peculiarly deficient in enzyms. It has even been claimed that they were the only bacteria devoid of catalase; no proteolytic enzyms have been demonstrated in them, and heretofore none acting on carbohydrates with the formation of acid. It is very probable that various classes of enzyms are formed by the lactic bacteria. As has been shown, a considerable part of the mass of the cheese consists of the cells of these organisms, which slowly disintegrate and their intracellular products are set free. It is not at all improbable that these products are the casual agents of changes that occur in the cheese, and that the rôles of the lactic bacteria are not limited to those previously mentioned.

OTHER GROUPS OF BACTERIA IN CHEDDAR CHEESE.

It is difficult to conceive that the varied chemical changes that occur during the ripening of Cheddar cheese can be due directly or indirectly to the lactic bacteria alone. It seems as though other biological factors must be operative, but, as was previously stated, no one has demonstrated the constant occurrence in large numbers of any other group of bacteria than the lactic group in Cheddar cheese. With the purpose of making a more complete examination of the cheese during the entire ripening period, the plate-culture work has been supplemented by other methods.

DETERMINATIONS BY MILK-DILUTION AND PLATE-CULTURE METHODS.

Since no solid medium seemed to promise better results than the standard media hitherto employed, milk was chosen as the medium most likely to permit of the growth of other groups of bacteria possibly present. In a previous publication[1] data concerning the distribution of a group of rod-shaped lactic bacteria in milk and other dairy products have been given. Among these organisms are included those found in many fermented milks, the *Bacillus bulgaricus* group, also the *B. casei* group, to which the work of Von Freudenreich and Jensen has attracted attention, as well as many of the acidophilous organisms found especially in the alimentary tracts of animals. These organisms were found to be constantly present in milk, butter, and in all the samples of Cheddar cheese examined. No quantitative analyses of cheese were made, however, in the work to which reference has been made.

[1] Hastings, E. G.; Hammer, B. W.; and Hoffman, C. Studies on the bacterial and leucocyte content of milk. Wisconsin Agricultural Experiment Station, Research Bulletin 6. Madison. June, 1909.

Von Freudenreich's work showed the constant presence in large numbers of the lactic bacilli in Emmental cheese. In the making of this cheese they are added to the milk in great numbers in the natural whey rennet employed, and the high temperature of the curd during the pressing of the cheese favors their development. These organisms find a more favorable condition for growth in milk than in any of the usual media, indeed some of the members of the group can not be cultivated except in milk.

With the idea of determining the number of the organisms of this group in cheese, inoculations in varying dilutions were made from cheese emulsions into flasks of sterile milk, which were protected from evaporation by tin-foil caps and incubated at 37° C. The dilutions increased by a ratio of 10, thus flasks of milk in the case of a single cheese might be inoculated with amounts of a cheese emulsion representing 0.001, 0.0001, and 0.00001 gram. The extent to which the dilutions were carried depended on the age of the cheese. An attempt was constantly made to carry the dilutions to a point where some of the flasks would remain sterile. The method is thus a quantitative one for bacteria that will grow under the conditions obtaining. The dilution method is to be considered a rough way of determining the number of certain classes of bacteria.

After incubation for one month at 37° C., the acidity of each flask was determined. The *Bacterium lactis acidi* group of organisms produce in average milk an acidity ranging from 0.7 to 1.25 per cent. The lactic bacilli produce an acidity usually exceeding 1.25 per cent. It has been the practice to infer that every flask of milk showing an acidity above 1.25 per cent after one month's incubation contains the lactic bacilli, either in pure culture or mixed with organisms of the *Bacterium lactis acidi* group. In the flasks showing an acidity less than 1.25 per cent it can not be inferred that the lactic bacilli are absent, since some cultures are found that produce no more acid than *Bacterium lactis acidi.* Many microscopical examinations were made of the flasks to establish the accuracy of the conclusions drawn from the data obtained by titrations. Microscopic examinations were also made of all the flasks from which the titrations did not give conclusive evidence concerning the organisms present. The smears were stained with Gram's stain and decolorized with a mixture of one part of anilin oil and two parts of xylol. This method of decolorizing removes the stain from the casein, but not from any of the organisms that have been found in cheese.

The dilution method thus gives information concerning the absolute and relative number of acid-forming bacteria of these two groups. It may also give information concerning the presence of still other types of bacteria when they are present in greater numbers than those of the groups already referred to, since they will then appear in the flasks in pure culture.

In Table 15 are given the results of the analysis of four cheeses. A portion of the data has been presented in Table 2 and is here repeated for ease of comparing the numbers of bacteria as determined by plate-culture methods and by the dilution method. It will be noted that the dilution method, as a rule, gives higher results than the plate culture. Out of 49 determinations, the dilution method gave higher results than the lactose-agar plate determinations in 30 cases. It is probable that the dilution method always gives higher results. If the results show a growth in a dilution of 1 to 10 millions, while the dilution of 1 to 100 millions gives a negative result, it can only be asserted that the bacterial content of the cheese was between 10 and 100 millions. It will be noted that the dilution method often shows many fold more bacteria than the plate cultures, and that the reverse is not often true. In cases where the latter gives the higher number, the excess is not great, only three or four times greater. The only explanation that can be given of the higher numbers obtained by the dilution method is that some types of bacteria present in the cheese that do not appear on the plate cultures find conditions favorable for growth in the milk. The results obtained by the dilution method indicate that other organisms than the *Bacterium lactis acidi* group are present in the cheese, undoubtedly in great numbers.

TABLE 15.—*Number of bacteria per gram of cheese as determined on lactose agar and gelatin plates, and in flasks of milk inoculated with high dilutions of cheese.*

[Numbers expressed in millions.]

Age.	Cheese No. 1.			Cheese No. 54.			Cheese No. 91.			Cheese No. 92.		
	Lactose agar.	Gelatin.	Milk dilution.	Lactose agar.	Gelatin.	Milk dilution.	Lactose agar.	Gelatin.	Milk dilution.	Lactose agar.	Gelatin.	Milk dilution.
Days.												
2							150		1,000	250	150	100
4	3		10						10,000			1,000
8							61	120	1,000	400	340	100
11	8	14	10						100			100
14				32		1,000	1,400	530	10,000	500		1,000
22	2.5	10	1,000				58	1,600	1,000	35		10,000
30	2	10	1,000	15	70	10						
37				32		100	270	480	1,000			1,000
45	40	25	10				1,400		100	340	100	1,000
56				32	51	10	1,300	250	100	225	225	10
77				51	40	1,000						
86	36.5	24	10				128	320	100	250	84	100
100				8.5	12	10	74	65	100	100	120	1,000
113	5	5	10				145	142	100	132	87	1,000
124				14	12	10	50	28.5	100	145	96	100
143	13	13	10	22.5	12.5	100	18	23.5	100	38.5	38	10
155							15	10.5	10	63	32	100
165							17.5	5.5	100	9	5.5	10
176	3.5	2.5	1									
187				6		1	7.5		10	12.5	2	100
208	2	1.5	10	1		100						

From the results obtained from the determinations of the degree of acidity attained by the flasks of milk inoculated with varying

amounts of cheese emulsion, and from the microscopic examinations of the same flasks, it has been possible to determine the relations existing between the different groups of acid-forming organisms, as well as their absolute numbers.

Table 16 has been constructed from such data. For ease in comparison the same data are expressed in Table 17 in percentages, and two additional cheeses are also included in the latter table. It will be noted from the data presented in Table 17 that during the early part of the ripening period the organisms of the *Bacterium lactis acidi* group make up over 90 per cent, and in many cases approximately 100 per cent, of the acid-producing flora of the cheese. With increasing age the ratio changes, until late in the ripening period the rod-shaped lactic bacilli predominate, and in many cases make up over 90 per cent of the acid-forming bacteria found in the cheese.

It will also be noted from the data given in Table 15 that the period at which the maximum number of bacteria is found, as determined by the dilution method in milk, is coincident with the appearance of the lactic bacilli, as shown in Table 16. Using any culture medium that furnishes favorable conditions for the growth of both groups of lactic bacteria, the maximum number of organisms should be found at the time when the group first to appear—the *Bacterium lactis acidi* group—has attained its maximum development, but before any considerable number of the cells have died, and at the time when the second group of organisms finds favorable conditions for growth in the cheese.

TABLE 16.—*Numbers of Bacterium lactis acidi and of acid-producing bacilli in cheese as determined in milk cultures.*

[Numbers expressed in millions.]

Age.	Cheese No. 1.		Cheese No. 54.		Cheese No. 85.		Cheese No. 91.		Cheese No. 96.	
	Bact. lactis acidi.	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.
Days.										
2	.....	.....	.....	.....	.....	.....	1,000	.....	1,000	.....
4	10	1	.....	.....	1,000	.....	10,000	10	100	.....
8	.....	.....	.....	.....	.....	.....	1,000	100	1,000	.....
11	10	1	.....	.....	1,000	10	100	.....	100	.....
14	.....	.....	1,000	10	1	10	10,000	1	10,000	1
22	1,000	1,000	.....	.....	.....	.....	1,000	100	1,000	1
30	100	100	10	.....	10	10	.....	.....	.....	.....
37	.....	.....	10	10	100	1	1,000	100	100	10
45	10	10	.....	.....	10	100	100	100	1,000	1
56	.....	.....	10	1	1,000	10	100	100	100	10
77	.....	.....	100	1	100	1	.....	.....	100	1
86	1	10	.....	.....	.....	10	100	100	100	10
100	.....	.....	10	1	10	10	100	100	100	10
113	.....	1	.....	.....	10	10	100	10	100	10
124	.....	.....	10	1	10	10	100	10	100	1
143	10	10	100	10	.....	10	100	10	100	1
155	.....	.....	.....	.....	10	1,000	10	10	10	10
165	.....	.....	.....	1	.....	.....	100	100	.....	10
176	1	1	.....	.....	1	100	.....	.....	.....	.....
187	.....	.....	1	1	10	10	.....	10	.....	10
208	.....	10	.....	100	.....	.....	.....	.....	.....	.....

TABLE 17.—*Relative proportion of the Bacterium lactis acidi type and the lactic acid-producing bacilli, as determined by dilution cultures in milk.*

Age.	Cheese No. 1.		Cheese No. 54.		Cheese No. 58.		Cheese No. 85.		Cheese No. 91.		Cheese No. 92.		Cheese No. 96.	
	Bact. lactis acidi.	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.
Days.	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
4	90.9	9.1	.....	.....	.....	.....	.....	.....	99.9	0.1	99.9	0.1	.....	.....
8	.....	.....	.....	.....	.....	.....	.....	.....	90.9	9.1	99	1	.....	.....
11	90.9	9.1	.....	.....	.....	.....	99	1	.....	.....	99.9	.1	.....	.....
14	.....	.....	99	1	90.9	9.1	.....	.....	99.99	.01	99.9	.1	99.99	0.01
22	50	50	.....	.....	.....	.....	9.1	90.9	90.9	9.1	99.9	.1	99.9	.1
30	50	50	99	1	.....	.....	50	50	.....	.....	.....	.....	.....	.....
37	.....	.....	50	50	.....	.....	99	1	90.9	9.1	90.9	9.1	90.9	9.1
45	50	50	.....	.....	.....	.....	9.1	90.9	50	50	90.9	9.1	99.9	.1
56	.....	.....	90.9	9.1	50	50	99	1	50	50	50	50	90.9	9.1
77	.....	.....	99	1	50	50	99	1	.....	.....	90.9	9.1	99	1
86	9.1	90.9	.....	.....	.....	.....	50	50	50	50	50	50	90.9	9.1
100	.....	.....	90.9	9.1	.....	.....	50	50	50	50	90.9	9.1	90.9	9.1
113	.....	.....	.....	.....	.....	.....	50	50	90.9	9.1	50	50	90.9	9.1
124	.....	.....	90.9	9.1	9.1	90.9	50	50	90.9	9.1	90.9	9.1	99	1
143	50	50	90.9	9.1	9.1	90.9	1	99	90.9	9.1	50	50	99	1
155	.....	.....	.....	.....	.....	.....	.....	.....	50	50	90.9	9.1	50	50
165	.....	.....	1	99	1	99	1	99	50	50	50	50	1	99
176	50	50	.....	.....	.....	.....	1	99	.....	.....	.....	.....	.....	.....
187	.....	.....	50	50	99	.....	50	50	1	99	1	99	1	99
208	.....	.....	.1	99.9	1	99	.....	.....	.....	.....	.....	.....	.....	.....

Since some of the lactic bacilli develop on lactose agar plates, it was thought that if all of the colonies on a certain portion of each plate were inoculated into milk one should obtain information concerning the sequence of development of the different groups of lactic bacteria in the cheese and also concerning the ratio existing between them.

For this purpose an area showing well-isolated colonies has been circumscribed, and every colony within the area has been inoculated into milk. It has been inferred that all tubes that curdled within 48 hours at 37° C. contained *Bacterium lactis acidi*, while those that curdled between the second and tenth day contained the lactic bacilli. Enough control work was done to show that this method of differentiation is sufficiently accurate for the purpose in hand. The tubes that did not curdle in 10 days were examined microscopically to determine the organism present.

Six cheeses have been thus examined at frequent intervals during the period of ripening. The data are given in Table 18. It will be noted that the results are confirmatory of those obtained by the dilution method in milk, although not so striking, since many of the lactic bacilli do not develop on the plate cultures. The period at which the lactic bacilli appear is later than in the previous examinations (Table 16), which demonstrated their presence in considerable numbers within the first week of the ripening period. The previous examinations were made with dilution cultures in milk. In

such the lactic bacilli may make themselves evident when they are present in very small numbers as compared with the *Bacterium lactis acidi.* With the plate-culture method, unless they are present in considerable numbers, their presence in the cheese is not likely to be detected. This error in the method has been previously pointed out. In the plate cultures they are likely to be missed unless they are present in a ratio of 1 to 10 of *Bacterium lactis acidi.*

The same cheeses have also been examined by the dilution method in milk. The results are given in Table 19. It will be seen that they are confirmatory of the previous analyses.

The great delicacy of the dilution method as a means of detecting the lactic bacilli in the presence of *Bacterium lactis acidi* is shown in the results given in Table 19. On the examination of cheese 308C at two days the percentage of *Bacterium lactis acidi* is given as 99.9999 and that of lactic bacilli as 0.0001. By this is meant that *Bacterium lactis acidi* was detected in dilutions approximately one million times greater than the lactic bacilli. For example, the acidity of the flask inoculated with one ten-thousandth gram of cheese was 1.64 per cent, thus indicating the presence of lactic bacilli, while the flask inoculated with one ten-billionth gram of the cheese had an acidity of 0.87 per cent, indicating *Bacterium lactis acidi.*

TABLE 18.—*The relative proportions of Bacterium lactis acidi and lactic bacilli in cheese as determined by plating on lactose agar.*

Age.	Cheese No. 307C.		Cheese No. 308C.		Cheese No. 309C.		Cheese No. 310C.		Cheese No. 311C.		Cheese No. 312C.	
	Bact. lactis acidi.	Lactic bacilli.	*Bact. lactis acidi.*	Lactic bacilli.	*Bact. lactis acidi.*	Lactic bacilli.	*Bact. lactis acidi.*	Lactic bacilli.	*Bact. lactis acidi.*	Lactic bacilli.	*Bact. lactis acidi.*	Lactic bacilli.
	P. ct.	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
Milk before rennet was added	100	0	100	0	100	0	100	0	100	0	100	0
When put in press	100	0	100	0	100	0	100	0	100	0	100	0
When taken from press	100	0	100	0	100	0	100	0	100	0	100	0
2 days	100	0	100	0	100	0	.....	.....	100	0	100	0
3 days	100	0	100	0	.....	.....	.....	.....	100	0	.....	.....
4 days	100	0	86	14	90	10	100	0	100	0	.....	.....
5 days	100	0	.....	.....	100	0	100	0	.....	.....	.....	.....
6 days	.....	.....	100	0	.....	.....	.....	.....	.....	.....	.....	.....
7 days	100	0	100	0	100	0	100	0	100	0	.....	.....
9 days	100	0	86	14	100	0	100	0	100	0	.....	.....
11 days	100	0	90	10	100	0	.....	.....	.....	.....	100	0
14 days	100	0	100	0	90	10	100	0	100	0	86	14
18 days	100	0	80	20	100	0	33	66	90	10	.....	.....
21 days	100	0	100	0	100	0	.....	.....	.....	.....	.....	.....
22 days	.....	.....	.....	.....	70	30	100	0	.....	.....	.....	.....
28 days	100	0	86	14	.....	.....	.....	.....	90	10	0	100
33 days	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
35 days	100	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
42 days	100	0	.....	.....	89	11	.....	.....	.....	.....	.....	.....
49 days	100	0	29	71	.....	.....	.....	.....	.....	.....	.....	.....
56 days	90	10	40	60	71	29	.....	.....	.....	.....	.....	.....
64 days	90	10	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

TABLE 19.—*The ratio between the numbers of Bacterium lactis acidi and the lactic bacilli at different stages in the ripening of Cheddar cheese, as determined by milk-dilution method.*

Age.	Cheese No. 307C.		Cheese No. 308C.		Cheese No. 309C.		Cheese No. 310C.		Cheese No. 311C.		Cheese No. 312C.	
	Bact. lactis acidi.	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.	*Bact. lactis acidi.*	Acid-producing bacilli.
Milk before rennet was added	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.* 99.99	*Per ct.* 0.01	*Per ct.*	*Per ct.*	*Per ct.* 99	*Per ct.* 1	*Per ct.* 99	*Per ct.* 1
Just before put in press			99.999	.001	99.9	.1			99.99	.01		
When taken from press	99.99	0.01	99.99	.01	99.9	.1					99	1
2 days	99.99	.01	99.9999	.0001	99	1	99.9	0.1	99	1	99	1
3 days	99.9	.1	99	1			50	50	90.9	9.1		
4 days	99.99	.01	99.9	.1	99.9	.1	99	1	99.99	.01	99	1
5 days	99.99	.01			99	1	99.99	.01				
6 days			90.9	9.1	99.9	.1					99.9	.1
7 days	90.9	9.1	90.9	9.1	99	1	99.9	.1	90.9	9.1	90.9	9.1
9 days	50	50	90.9	9.1	50	50	99	1	99	1		
11 days	99	1	50	50	50	50					90.9	9.1
14 days	99	1	90.9	9.1	90.9	9.1	90.9	9.1	50	50		
18 days	50	50	50	50	50	50						
21 days	90.9	9.1	99	1	90.9	9.1						
28 days	50	50	90.9	9.1	50	50						
33 days					50	50						
35 days	50	50	50	50								
42 days	50	50	99	1	90.9	9.1						

It must not be inferred that the figures given in the tables representing the results obtained by the dilution method indicate the exact number of the different groups of lactic organisms or the exact ratio existing between them. The sudden increase or decrease of the ratio is due to the inherent errors of the dilution method. It would be an endless task to show by this method, or any other, in fact, the exact proportion between the different types of organisms in the cheese at various stages in the ripening period.

From the data presented there would seem to remain no doubt that the lactic bacilli develop later than the *Bacterium lactis acidi* group, making their appearance within a week or 10 days after the cheese is made, gradually increasing in numbers and probably attaining their maximum during the first month and then gradually decreasing in numbers. The number found per gram of the cheese ranges from a few million to one billion. Usually they do not attain such great numbers as do the ordinary lactic bacteria. Again, their numbers may equal the lactic bacteria, as in cheese No. 1, Table 16.

DETERMINATIONS BY MICROSCOPIC EXAMINATION OF THE CHEESE.

It was also thought that the gradually changing flora should manifest itself in the appearance of microscopic preparations made from the cheese. At the various periods of sampling smear preparations

were made from the emulsions and stained with Gram-Weigert's stain as described on page 32.

The preparations show in a general way the same change in flora as has already been made evident by the analyses presented. It is not to be expected that the microscopic examination would give results as striking as the cultural, since the latter measures the living cells, the former only those cells that have not lost their staining properties.

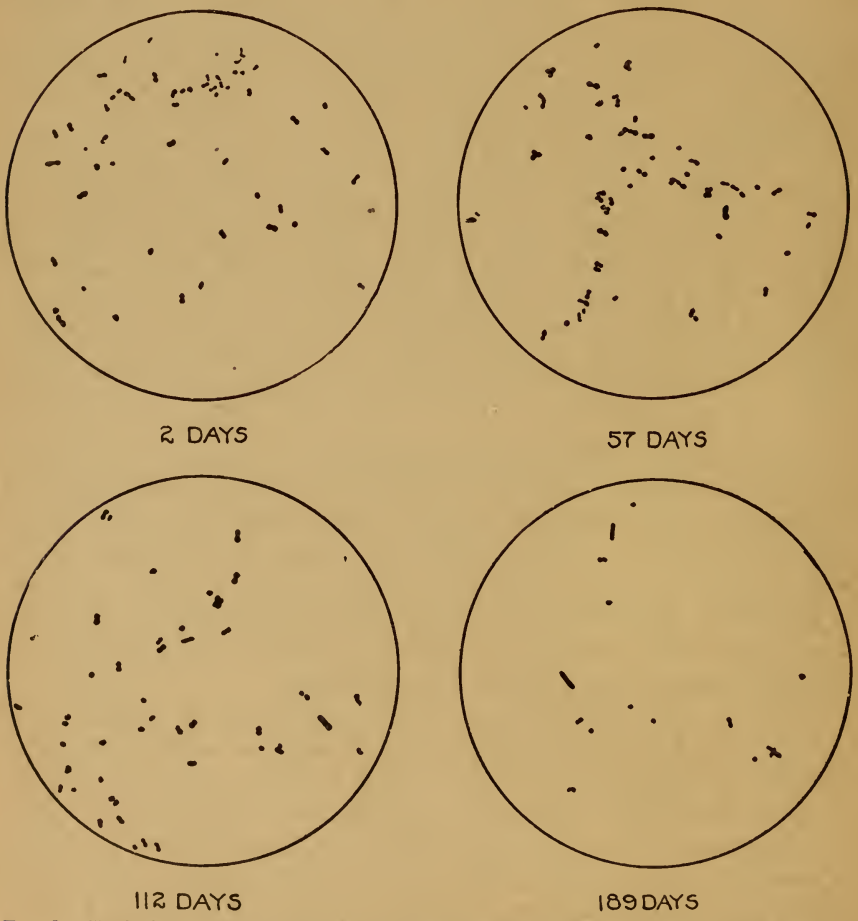

FIG. 3.—Typical microscopic fields from cheese No. 54 at different stages in the ripening process. (Camera lucida drawings.)

Figures 3 and 4 have been prepared from camera-lucida drawings of typical microscopical fields of several slides made from 2 cheeses. The results of the cultural examination have been presented in Tables 15 and 16. Figure 3 shows that at 2 and 57 days the organisms are almost, if not wholly, of *Bacterium lactis acidi* group; at 112 days there is a preponderance of *Bacterium lactis acidi* and a few lactic bacilli, while at 189 days but few *Bacterium lactis acidi* cells

remain, the lactic bacilli having become more evident. Figure 4 indicates likewise that at 29 and 78 days the organisms are wholly of the *Bacterium lactis acidi* group, while at 121 and 185 days a decrease is seen in *Bacterium lactis acidi* and an increase in lactic bacilli. It will be noted that rod-shaped organisms do not appear nearly as soon as would be indicated by the cultural analyses, but that they do at last appear, especially when the cells of the lactic bacteria have greatly decreased in numbers. This may again be taken as evidence

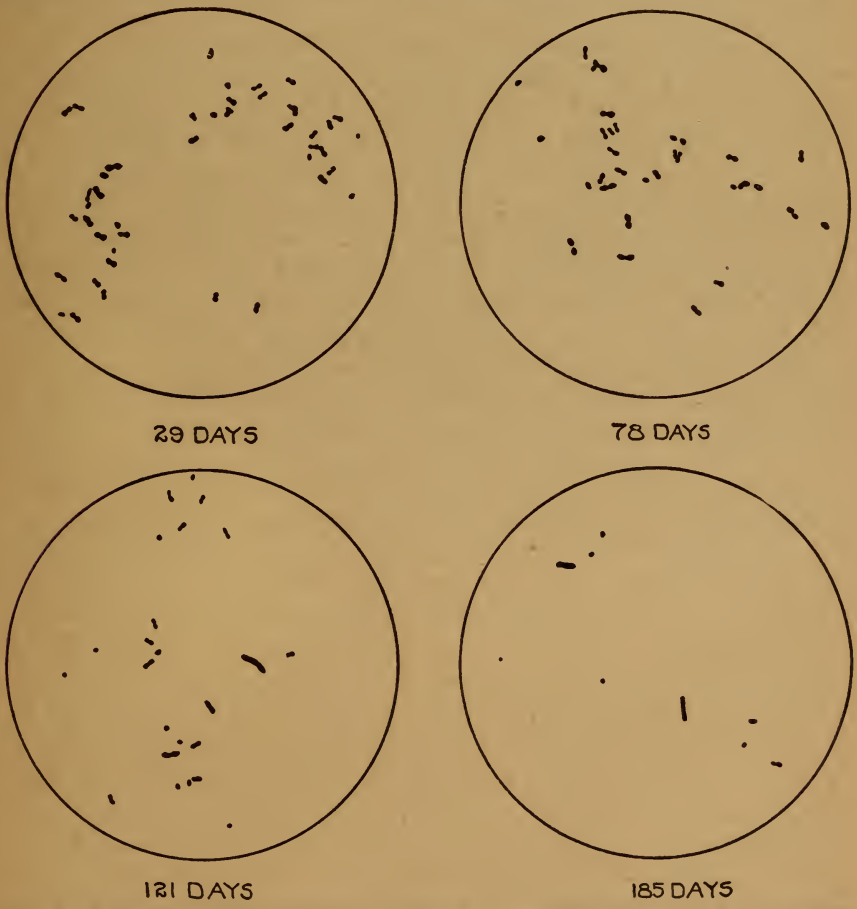

FIG. 4.—Typical microscopic fields from cheese No. 91 at different periods in the ripening process. (Camera lucida drawings.)

that the lactic bacilli develop subsequent to the *Bacterium lactis acidi* group and that they tend to disappear less rapidly.

DISAPPEARANCE OF BACTERIAL CELLS IN CHEESE.

The drawings presented in figures 3 and 4 are evidence of the gradual disappearance and disintegration of the bacterial cells in cheese. It is a well-known fact that the enzyms elaborated by bacteria in pure cultures continue to act long after the death of the cells. There

are many reasons for believing that there is no enzym action until cell disintegration begins. It has been previously shown that the *Bacterium lactis acidi* group of bacteria elaborate enzyms that are able to increase the acidity of milk. As previously indicated, it may be surmised that still other types of enzyms are formed by this group of bacteria. The direct influence of the immense number of acid-forming bacteria, amounting to billions per gram, must be of great importance in cheese ripening; the indirect action through their enzyms may be still greater.

It is certainly true that in normal Cheddar cheese the period of development of the ordinary lactic bacteria—the organisms which heretofore have been believed to be the only ones of importance in cheese because of their constant presence in great numbers—is followed by the development of another group of organisms, which, while they ferment milk sugar, producing lactic acid, must have some other source of carbon in cheese on account of the total disappearance of the sugar before their maximum period of development. The influence of the first group has been pointed out early in this paper. The rôle of the second group can not at this time be determined, but because of their constant presence in numbers, approximating if not equaling those of the first group, their influence on the ripening of the cheese can not be a minor one.

DETAILED STUDY OF LACTIC BACILLI.

The work of Von Freudenreich and numerous later investigators indicates that the lactic bacilli represent a group with certain common characteristics, such as morphology, optimum temperature for growth, and to some extent in the production of compounds from the sugar fermented. In all fields of bacteriology it is difficult at the present time to draw the boundary lines of any group of bacteria. The placing of the lactic bacilli found in cheese in a single group may be incorrect, but because of the fact that all the cultures studied have certain characters in common, it seems best to discuss them as a single group, although more detailed study might divide them into a number of groups. A comparative study has been made of 20 pure cultures isolated in the course of the work. The main facts of this study are here presented. All the cultures were isolated from cheese with the exception of No. 160, which was obtained from milk. Cultures 58A and 58B represent different colonies from plates made to insure the purity of the cultures to be used in the detailed study. The differences noted in the study of these two cultures originally from the same culture illustrate the differences one may expect to obtain in cultural work.

The action of the different cultures in milk is given in Table 20. in which they have been arranged according to the degree of acidity produced in milk.

ACIDITY PRODUCED.

It will be noted from the data in Table 20 that both the rate of acid development, and hence the time required to curdle milk, as well as the maximum amount of acid produced, varies widely, the acidity varying from 0.91 per cent to 2.31 per cent. The acidity produced by each culture increased after the tenth day. Since the flasks were protected by tin-foil caps, this increase can not have been due to evaporation.

White and Avery,[1] in studying cultures from fermented milks, noted the same differences in acid production. They established two groups, one producing an acidity in milk of about 1 per cent, the other of 3 per cent, in 10 days. Such a division of the cultures studied could not be made.

Rogers[2] states that a typical culture of the lactic bacilli from fermented milk produces nearly 3 per cent of acid in three days at 37° C. Von Freudenreich and Thöni[3] studied cultures from Emmental cheese that produced from 0.2 to 1.26 per cent of acid in whey to which peptone had been added.

Out of the several hundred titrations made of milk inoculated with cheese emulsions but 15 showed an acidity above 2 per cent. When raw milk is incubated at 37° C., the acidity will often, if not in the majority of cases, exceed 2 per cent and at times may reach 3.5 to 4 per cent. It would thus seem that certain lactic bacilli present in milk do not find favorable conditions for development in Cheddar cheese.

FORMS OF LACTIC ACID PRODUCED.

A number of investigators have determined the rotary power of the lactic acid formed by the lactic bacilli. Bertrand and Weisweiller[4] concluded that the acid was a mixture of the levo and dextro forms, with a slight predominance of the latter.

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[1] White, Benjamin, and Avery, Oswald T. Observations on certain lactic acid bacteria of the so-called Bulgaricus type. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 25, No. 5/9, pp. 161–178. Jena, Nov. 30, 1909.

[2] Rogers, L. A. Fermented milks. United States Department of Agriculture, Bureau of Animal Industry, Twenty-sixth Annual Report (1909), pp. 133–161. Washington, 1911. Reprinted as Bureau of Animal Industry Circular 171, Washington, 1911.

[3] Von Freudenreich, Edward, and Thöni, J. Sur l'action de différents ferments lactiques sur la maturation du fromage. Revue Générale du Lait, vol 4, No. 8, pp. 169–181, Jan. 30; No. 9, pp. 200–209, Feb. 15; No. 10, pp. 225–232, Feb. 28; No. 11, pp. 247–259, Mar. 15. Lierre, 1905.

[4] Bertrand, Gabriel, and Weisweiller, Gustav. Action du ferment bulgare sur le lait. Annales de Institut Pasteur, vol. 20, No. 12, pp. 977–990. Paris, Dec. 25, 1906.

TABLE 20.—*Action of lactic bacilli in milk.*

Culture No.	Acidity produced in milk.		Rotation of acid.	Time of curdling.	Culture No.	Acidity produced in milk.		Rotation of acid.	Time of curdling.
	10 days.	45 days.				10 days.	45 days.		
	Per ct.	*Per ct.*		*Days.*		*Per ct.*	*Per ct.*		*Days.*
5	0.324	0.91	.....	11	116.2	1.2	1.44	Inactive.....	.....
152	.643	1.12	Inactive.....	.....	92	1.24	1.4	.....	4
52	.68	1.05	.....	9	160	1.37	1.6	Dextro.....	4
165.1	.69	1.10	.....	.....	165	1.41	1.57	.....	4
163	.....	1.26	.....	9	113	1.57	1.88	Dextro.....	4
58A	.89	1.38	Inactive+levo ..	.....	71	1.6	1.75	.....	4
58B	.86	1.21	.....do.....	.....	91	1.68	.....	.....	4
128	.89	1.2	.....	.....	85	1.73	1.89	Dextro+inactive	4
116	.81	1.36	Inactive.....	4	116.1	1.75	1.96	.....	.....
120	1.02	.....	.....do.....	.....	96	1.89	2.31	.....	3
104	1.17	1.46	Dextro+inactive	4					

Heinemann and Hefferan[1] report the formation of an inactive acid, while White and Avery[2] found that the organisms that produced a large amount of acid, 3 per cent, formed an inactive acid, whereas the cultures that formed acid slowly and in smaller amounts showed a levo-rotary power at the end of 24 hours, but in older cultures there were approximately equal amounts of levo and of inactive acids.

The rotary power of 9 of the cultures studied was determined by Dr. J. M. Currie. Four of these produced inactive acid; 1 produced inactive and levo acid, with a predominance of the inactive form; 2 produced a mixture of dextro and inactive acids, with a predominance of the dextro-rotatory form; and the remaining 2 produced pure dextro acid. One culture isolated from milk, of which no other study was made, produced pure levo acid. With 11 cultures isolated from other sources than milk or cheese, only the pure dextro or pure inactive acids were found.

This type of organism, then, can produce inactive acid, or active acid of either modification, or a mixture of the inactive acid with either of the active acids. All of these forms were found in cultures isolated from Cheddar cheese, with the exception of the pure levo acid.

TYPE OF CURD PRODUCED.

In most of the more active cultures the reduction of litmus and the return of color proceeded in exactly the same manner as in a litmus-milk culture of *Bacterium lactis acidi.* On the other hand, curdling took place with only a slight or partial reduction of the litmus in some cultures.

[1] Heinemann, P. G., and Hefferan, Mary. A study of Bacillus Bulgaricus. Journal of Infectious Diseases, vol 6, No. 3, pp. 304–318. Chicago, June 12, 1909.

[2] White, Benjamin, and Avery, Oswald T. Observations on certain lactic acid bacteria of the so-called Bulgaricus type. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 25, No. 5/9, pp. 161–178. Jena, Nov. 30, 1909.

There was a slight gas formation in most of the cultures, evidence of which was found in the slight furrows or tiny holes which sometimes appeared in the curd. These gas holes were, however, not always found in curds from the same culture. Although most of the organisms grew in lactose-agar shake cultures, no gas formation was noted in any case.

FERMENTATION OF SUGARS.

Sixteen of the cultures were tested as to their power to ferment sugars. The cultures were incubated eight days at 37° C. The test for growth and fermentation of the sugar was the reddening of litmus paper. The results are given in Table 21. All of the strains tested except one, No. 128, grew in glucose bouillon. Many of the strains made no growth or only a feeble growth (indicated by the sign ±) in maltose and sucrose bouillon, although all except No. 128 grew in one or the other, or both, of these sugars.

TABLE 21.—*Growth of lactic bacilli in culture media.*

Culture No.	Growth in sugar bouillon.			Growth on lactose agar.	Growth on ordinary gelatin.	Culture No.	Growth in sugar bouillon.			Growth on lactose agar.	Growth on ordinary gelatin.
	Glucose.	Maltose.	Sucrose.				Glucose.	Maltose.	Sucrose.		
5	.....	.....	.....	+	−	116.2	+	±	+	+	−
152	+	+	+	+	+	92	.....	.....	.....	+	−
52	+	.....	−	+	+	160	+	−	±	+	−
165.1	+	+	+	±	−	165	+	+	+	+	−
163	.....	.....	.....	+	−	113	+	+	+	+	−
58A	+	+	±	+	+	71	+	+	±	+	−
58B	+	+	−	+	+	91	+	+	+	+	−
128	−	−	−	−	−	85	+	+	+	+	+
116	+	+	−	+	+	116.1	+	+	+	+	−
120	+	−	+	+	+	96	.....	.....	.....	+	−
104	+	+	+	±	.....						

± indicates feeble growth.

FORM OF COLONIES.

The growth upon lactose agar and ordinary gelatin was determined in plate cultures on the same media that was used for the bacteriological analysis of cheese. Growth was obtained in lactose-agar plate cultures from every strain except No. 128. There was a good growth in most of the cultures, although with some only a few colonies developed. When the plates were incubated for several days the colonies continued to increase in size, sometimes attaining a diameter of 1 mm. The surface colonies are round and the deep colonies have the common flattened elliptical form. They can not be distinguished from the colonies of *Bacterium lactis acidi*. This is probably one reason why previous investigators have failed to find this type of organism in Cheddar cheese.

Growth upon plates of ordinary gelatin was obtained from 8 of the cultures. This, however, was not constant for some strains

which developed from one inoculation failed to grow from another inoculation. The variable growth of this type of organism in plate cultures can account for some of the inconsistent results obtained in the quantitative analysis of cheese by means of lactose-agar and gelatin plates.

The readiness with which some of these cultures of lactic bacilli isolated from cheese grow upon ordinary media is rather surprising, in view of what has been stated by many authors concerning this property. One of the characteristics generally given for this group of organisms is their meager growth upon ordinary media. The five cultures studied by Von Freudenreich failed to grow on ordinary gelatin or on gelatin to which an extract of cheese had been added. Cohendy [1] and Löhnis [2] found these organisms hard to cultivate because of their reluctance to grow upon the ordinary nutrient media. White and Avery state that when freshly isolated from their natural environment—milk—they do not develop on any of the usual nutrient media, even though sugar be present. Heineman and Hefferan, on the other hand, state that they grow well in milk, in media prepared from milk, or if glucose is added to the ordinary media. All of the cultures studied were isolated by means of lactose-agar plates with the exception of No. 128, which was obtained in pure culture in the dilution cultures in milk.

THERMAL DEATH POINT.

The resistance of the cultures to heat was determined on 3-day-old milk cultures which had been diluted and shaken with water and then drawn into sterile capillary tubes. The tubes were held for 30 seconds in water at various temperatures. Most of the cultures were killed at some temperature between 62° and 67° C. Two cultures were killed between 60° and 62.5° C., and two cultures were killed at about 70° C.

MORPHOLOGY.

The bacilli are nonmotile and do not bear spores. In a single microscopic field of a slide prepared from a pure culture the organisms may vary in length from 2.5 microns to 20 or 30 microns or more. Sometimes the filaments are very long. In one of the pure cultures a filament was found 75 microns in length. In one of the mixed cultures inoculated from cheese there was found a filament which was about 960 microns in length, extending four times across the field of

[1] Cohendy, Michel. Essais d'acclimation microbienne persistante dans la cavité intestinale. Comptes Rendus Hebdomadaires des Séances de la Société de Biologie, vol. 60 (année 58, tome 1), No. 7, pp. 364–366. Paris, Feb. 23, 1906.

[2] Löhnis, F. Versuch einer Gruppierung der Milchsäurebakterien. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 18, No. 4/6, pp. 97–149. Jena, Mar. 14, 1907.

the microscope. The filaments are apparently made up of a number of individual cells which for some reason do not show the cell divisions, for frequently chains of rods are found instead of filaments. Occasionally in a mixed culture from a cheese inoculation filaments were found very much curled, or curled at one end, but attempts to isolate a culture which would regularly produce curled filaments always resulted in securing a culture with the usual morphology. No curled filaments were ever observed in pure cultures. The width of the cells in a pure culture seems to be fairly constant; in some cultures the individual cells are all slender, in other cultures they are all comparatively thick. In different cultures the width varies from 0.4 to 1.3 microns. No branching forms have ever been observed.

When stained with methylene blue the ends of the cells may take the stain much more deeply than the remainder of the cell, or the deeply stained spots may be scattered irregularly in the filament. Occasionally the deeply stained spots appear as tiny nodules on the rod. These characteristic staining properties are less frequently exhibited when stained by the Gram-Weigert method. In a weakened culture filaments are sometimes found with some of the cells Gram positive, some of them Gram negative, and other cells taking the stain partially.

Although there is such a wide difference between the cultures in regard to the production of acidity in milk, the other characteristics do not differentiate a culture producing a low acidity from one which produces a high acidity. The morphology, however, seems to bear some relation to the acidity produced, those producing a high acidity being more slender than those producing a low acidity.

CONDITIONS FOR GROWTH IN CHEESE.

It has been pointed out that in their development in cheese the lactic bacilli follow the *Bacterium lactis acidi* group, and that the greater part of the growth must occur after the disappearance of the sugar. Since lactic acid is the principal by-product of the fermentation of the sugar, it might seem probable that the lactic bacilli would make use of this as a source of carbon and of energy. Analyses of cheese show no decrease in lactic acid [1] at the period of development of the lactic bacilli. This indicates that this group of bacteria does not act on the lactic acid.

The action of the pepsin of the rennet extract activated by the lactic acid results in the formation of peptones from the paracasein. The favorable effect of peptone on the lactic bacilli is shown in the

[1] Suzuki, S. K.; Hastings, E. G.; and Hart, E B. The production of volatile fatty acids and esters in Cheddar cheese and their relation to the development of flavor. Wisconsin Agricultural Experiment Station, Research Bulletin 11. Madison, June, 1910. See p. 135.

following experiment. Small flasks containing 100 c. c. of sterile milk were inoculated with cultures Nos. 5, 92, 91, and 96. To one flask of each set there had been added before sterilization 0.5 gram of peptone, and to another flask 1 gram. The control flask received no peptone. In Table 22 the acidity at 10 days and 45 days is given. The results show clearly that milk to which peptone has been added is a better medium than plain milk for the growth of this group of organisms. This was especially true in the case of culture No. 5, which usually curdled milk in about 11 days, but which curdled the milk to which peptone had been added in two days—more rapidly than the most active cultures curdle plain milk. The time of curdling was shortened by the peptone 3 days in the case of culture No. 91, and 1 day in cultures Nos. 92 and 96. In every case the final acidity at 42 days was considerably greater than in plain milk, and in every case but one (culture No. 91, at 42 days) there was a greater development of acidity in the milk containing 1 gram than in the milk containing 0.5 gram of peptone.

Incidentally, this experiment shows that the cessation of growth in milk when a certain percentage of acidity is reached, which is a fairly constant percentage for each particular culture, is not brought about by the antiseptic action of the acid, but by a lack of suitable nitrogenous food.

TABLE 22.—*Percentage of acidity produced by lactic bacilli in milk to which peptone has been added.*

Culture No.	Acidity in 10 days.			Acidity in 42 days.		
	Plain milk.	Milk with 0.5 gram peptone.	Milk with 1 gram peptone.	Plain milk.	Milk with 0.5 gram peptone.	Milk with 1 gram peptone.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
5	0.324	1.19	1.32	0.855	1.35	2.34
92	1.24	1.4	1.58	1.4	1.56	1.77
91	1.68	1.9	2.1	.....	2.42	2.29
96	1.58	1.87	1.96	1.93	2.13	2.25

The action of the enzyms present in the cheese curd brings about an increasing amount of soluble nitrogenous compounds so that after one month of ripening about 18 per cent of the total nitrogen is in the form of water-soluble compounds.[1] This action must render the curd a medium favorable to the growth of the lactic bacilli, and particularly to those strains similar to culture No. 5, which grow so slowly in milk, or fail to grow therein, as frequently happens.

The dependence of this group of organisms upon enzyms from other sources is shown by growing them together with other types

[1] Van Slyke, L. L., and Hart, E. B. Conditions affecting chemical changes in cheese-ripening. New York Agricultural Experiment Station, Bulletin 236. Geneva, July, 1903. See p. 150.

of bacteria. Marshall and Farrand[1] have shown that when *Bacterium lactis acidi* grows in milk together with certain other organisms there is an associative action which accelerates the production of acid and the proliferation of cells. This quickening action was found to occur in the case of 57 per cent of the cultures grown in association with *Bacterium lactis acidi*. A similar action, but more pronounced, takes place when the lactic bacilli grow in milk together with certain other types of organisms.

Suspensions in water were made of a 48-hour milk culture of the lactic bacillus No. 104 and also of 48-hour glucose bouillon cultures of two different strains of liquefying bacteria, which were isolated from poor cheese. This particular type of organism has not been found in good cheese. Small flasks of milk were inoculated with various dilutions of these suspensions, as given in Table 23. Since the results from the two experiments were similar, the figures for only one of them are given.

TABLE 23.—*Associative action of lactic bacilli and a liquefying organism.*

Flask No.	Inoculation with liquefier.	Inoculation with lactic bacilli.	Acidity in 2 days.	Acidity in 3 days.	Acidity in 7 days.
			Per cent.	*Per cent.*	*Per cent.*
I	1:1,000	No inoculation	0.17	0.17	0.34
II	1:1,000	1:10,000,000	.18	.59	1.44
III	1:1,000	1:100,000	.26	.85	1.53
IV	1:1,000	1:1,000	.40	.96	1.44
V	1:100,000	1:1,000	.36	.91	1.42
VI	1:10,000,000	1:1,000	.42	.99	.....
VII	No inoculation	1:1,000	.31	.62	1.36

It will be seen that in 2 days the production of acidity was accelerated in the cultures which received an inoculation of the liquefier. This was more pronounced in 3 days, but less pronounced in 7 days, when the flask which received no inoculation of the liquefier showed only a small amount of acidity less than the flasks inoculated with both cultures.

Other experiments were made with cultures of coccus forms isolated from cheese. The liquefying action of these was not pronounced, yet they exerted a stimulating effect on the lactic bacilli. Reference to the coccus forms found in cheese will be made later.

SOLVENT EFFECT ON MILK PROTEINS.

Von Freudenreich was the first to demonstrate the fact that lactic bacilli exert a digestive effect. The same action[2] was shown for cul-

[1] Marshall, Charles E., and Farrand, Bell. Bacterial associations in the souring of milk. Michigan Agricultural Experiment Station, Special Bulletin 42. East Lansing, Mar., 1908.

[2] Hastings, E. G., Hammer, B. W., and Hoffman, C. Studies on the bacterial and leucocyte content of milk. Wisconsin Agricultural Experiment Station, Research Bulletin 6. Madison, June, 1909. See p. 202.

tures isolated from milk and has also been demonstrated by more recent investigators.

The solvent effect of 8 of the cultures studied has been determined by the analyses of milk cultures after 3 months' incubation. The results are given in Table 24.

TABLE 24.—*The solvent effect of lactic bacilli on milk proteins.*

Culture.	Soluble N. in 100 c. c. of milk.	Increase in soluble N in 100 c. c. of milk.	
	Gram.	*Gram.*	*Per cent*
Sterile milk	0.064	.....	....
116	.072	0.008	12.5
113	.076	.012	18.7
152	.080	.016	25.0
120	.081	.017	26.5
116.1	.090	.026	40.6
116.2	.092	.028	43.7
71	.096	.032	50.0
52	.104	.040	62.5

COCCUS FORMS IN CHEDDAR CHEESE.

Several investigators have mentioned coccus forms in relation to cheese ripening. Von Freudenreich and Thöni [1] found regularly a liquefying micrococcus in quite large numbers in fresh Emmental cheese. They made a number of experimental cheeses, using this type of organism alone as a starter or together with lactic starters. They concluded that when present in too great numbers the liquefying micrococci produced bitterness, but that they disappeared quite rapidly in practical cheesemaking. Gorini [2] isolated a coccus with the property of peptonizing casein in an acid medium from Grana, Emmental, and Edam cheese. He found these forms not only in the fresh cheese, but in cheese several months old. He thought that they were not all of the same type, and he related them to the normal flora of the udder. In a more recent publication [3] Gorini states that later investigations have confirmed his opinion that acid-producing peptonizing ferments are important in the ripening of cooked cheeses and that the fundamental flora of Grana and other cooked cheeses is composed of two types of bacteria; (1) lactic acid bacteria, and (2) acid-producing, peptonizing ferments. In the latter class he includes bacillus forms with those properties and divers types of cocci.

No reference to coccus forms having been found in considerable

[1] Von Freudenreich, Edward, and Thöni, J. Sur les bactéries du lait normal et leurs rapports avec la maturation des fromages. Revue Générale du Lait, vol. 2, No. 11, pp. 241–247, Mar. 15; No. 12, pp. 271–280, Mar. 30, Lierre, 1903.

[2] Gorini, C. Sur la présence de bactéries productrices d'acidité et de présure dans les fromages en maturation. Rue Générale du Lait, vol. 3, No. 22, pp. 505–510. Lierre, Aug. 31, 1904.

[3] Gorini, C. Studi sulla fabbricazione dei fromaggi Grana, ecc. Italy-Ministero di Agricoltura, Industriae Commercia. Bollettino. Anno 9, vol. 1, ser. C, No. 6, pp. 9–17. Rome, June, 1910.

numbers has been made in the literature on Cheddar cheese. Harding and Prucha [1] report the presence of acid-producing, liquefying coccus forms in 9 out of 10 cheeses studied. They state that these forms occurred sufficiently often to suggest that they might play some part in the ripening changes, but that they made little headway in the cheese, and their number, as compared to the total germ content of the cheese, was relatively insignificant.

Coccus forms which produce a small amount of acidity were occasionally found to be the predominating type of organism in the cheeses studied, as shown by the milk cultures from high dilutions of the cheese. Unfortunately, with the dilution method of analysis this type can be differentiated only when it predominates, for if it occurs in a culture with the other cheese organisms it can not be distinguished with certainty from the *Bacterium lactis acidi* type in a microscopic preparation, and the titration of the milk gives no information, since its production of acidity is less than either the lactic bacilli or *Bacterium lactis acidi.* However, this type has been found to predominate at some time or other in 11 of the 13 cheeses examined by this method.

In 4 of the 11 cheeses the maximum number of coccus forms found was 100,000,000 per gram, in 4 other cheeses 1,000,000,000, and in the 3 remaining 10,000,000,000 per gram. The time at which they predominated varied from the 14th to the 161st day. This would indicate that they increase early in the ripening period and maintain such numbers for a considerable period. The coccus forms have more or less of a liquefying action on gelatin; a few show a decided action, some none at all, while the majority produce a minute depression in the gelatin around the colony. Their action is not comparable with that of those organisms usually classed as liquefying bacteria. They produce small crystals in milk that enables one to differentiate them from *Bacterium lactis acidi.* The nature of these crystals has not yet been determined.

The various cultures produced acidities in milk varying from 0.35 to 0.80 per cent.

As has been previously mentioned, all of the colonies from a circumscribed area on the lactose-agar plates prepared from the cheeses last examined were inoculated into milk. The formation of the crystals was used to distinguish the coccus forms. In some cheeses, especially those with a low germ content, the cocci have made up from 10 to 40 per cent of the bacteria as determined by lactose-agar plate cultures. In other cheeses they have not been detected in many of the examinations made, and when found were in minor numbers.

[1] Harding, H. A., and Prucha, M. J. The bacterial flora of Cheddar cheese. New York Agricultural Experiment Station, Technical Bulletin 8. Geneva, Dec., 1908. See p. 184.

It should be mentioned, however, that this method of detecting the various kinds of organisms in cheese is subject to considerable error, especially when certain forms are present in much smaller numbers than other forms, as is the case with the coccus forms as compared with the lactic bacteria of both groups in many of the cheeses examined. The error can be reduced by making subcultures of a greater number of colonies. By the extension of this method or by the use of some differentiating medium they may be shown to make up a considerable part of the flora of normal Cheddar cheese.

CHROMOGENIC COCCI.

The chromogenic cocci can be distinguished on the plate cultures when they are present in appreciable numbers. In a portion of the work the number of colonies of chromogens appearing on gelatin plates was determined. The results seemed to indicate a relative increase late in the ripening period. As has previously been pointed out, it is impossible to determine, in the case of any group of organisms for which no means of differentiation from other groups has been found, whether growth is taking place in cheese or not.

It is characteristic of the chromogenic cocci that they persist and possibly grow under conditions which rapidly destroy less resistant types. They are present in butter, in which food material is limited, and in which the moisture represents a saturated solution of sodium chlorid. Efforts were made to determine their number in cheese by plating on gelatin containing 3 or 4 per cent of sodium chlorid. The results were quite satisfactory as far as the chromogenic types were concerned, but since many other coccus forms did not grow thereon its use was given up.

In some cheeses very few chromogenic forms have been found, while in others of similar quality they have been present quite consistently in considerable numbers. In cheese which contained more than the usual amount of salt they made up a relatively greater proportion of the flora than in other cheese.

LIQUEFYING ORGANISMS.

Attention has been directed to those organisms that show a pronounced liquefying action on gelatin and casein. The results have been confirmatory of a statement made earlier that they can not be considered of importance in the ripening of Cheddar cheese since they are not consistently present in sufficient numbers to exert any effect.

THE SEQUENCE IN DEVELOPMENT OF BACTERIAL GROUPS IN CHEDDAR CHEESE.

In the first part of this bulletin it was stated that the normal ripening of each kind of cheeese is to be looked upon as a problem in the ecology of microorganisms, and that the only group of organisms which has been found by previous investigators in every Cheddar cheese in great numbers is the *Bacterium lactis acidi* group. The work herein reported proves that another group of bacteria, the so-called *B. bulgaricus* group, develops after the first, and that it reaches approximately equal numbers.

Each of these groups of organisms produces certain changes in the cheese mass, consuming the same class of substances and giving out the same by-products in every cheese which ripens normally. The second group takes up the work of decomposition at a certain stage of the ripening, and brings about its own peculiar changes which prepare the cheese mass for possibly still other types of organisms, and so on to the end of the ripening.

If a certain sequence of groups of microorganisms is essential for the preparation of a certain product from raw material, and if the various members of the sequence find favorable conditions for growth in the raw material, the resulting product will depend on the first member of the normal sequence developing to the necessary degree before the second appears. If the first is overwhelmed by the addition of great numbers of the second, the decomposition changes will not be normal. The presence of great numbers of organisms of the first member of the sequence will not cause a disturbance in the normal decomposition changes. Thus, in the manufacture of Cheddar cheese, cultures of *Bacterium lactis acidi* are added.

The other essential groups of microorganisms are present in the milk in small numbers, but as conditions become favorable they develop in the cheese, and a typical Cheddar cheese results.

If heavy inoculations of lactic bacilli are made in milk which contains a small number of *Bacterium lactis acidi* the normal ecological balance will be destroyed, and the result will not be a normal product. Experiments have been made along this line by the use of milk which has been pasteurized in such a manner that the result has been the great reduction of all groups of bacteria therein, but the total destruction of none. In its decomposition such milk is similar to raw milk, which contains but few bacteria. If cheese is prepared from such milk after the addition of a culture of *Bacterium lactis acidi*, it may ripen in a quite normal manner. The development of the flavor is usually slower than in a cheese made from raw milk. If to this same milk is added a culture of lactic bacilli instead of *Bacterium lactis acidi*, the ripening is not nearly so normal. The result is an increased rate of ripening and the production of an abnormal flavor.

This work also indicates that it is often useless to attempt to establish the rôle of any organism in cheese ripening by the addition of cultures to the milk to be used, since thereby the natural equilibrium is destroyed, and the results obtained indicate that the addition has injured the product, and hence the conclusion is drawn that the organism added is not only not essential, but even harmful, although the organism may be an essential factor in the decomposition changes when developing in its normal sequence.

The constant presence in large numbers is the only certain proof of the importance of an organism or group of organisms in the ripening of any cheese.

SUMMARY.

1. From the same raw materials various kinds of cheese are prepared, which differ especially in flavor. The factors that determine whether a cheese to be prepared from a given mass of milk, rennet, and salt is to be of one kind or another are to be found in the methods of the cheese maker, who is able to vary in one way or another the composition of the cheese, with the result that conditions are established that favor or retard the growth of the groups of microorganisms, which must be the determining factors between different kinds of cheese.

2. The only group of bacteria found constantly in great numbers in Cheddar cheese by previous investigators is the *Bacterium lactis acidi* group. The functions of this group in Cheddar cheese are, through their chief by-product, lactic acid—

(*a*) To favor the curdling of milk by rennet.

(*b*) The bacteria of the milk are held in great part in the curd. Through the acid they influence the shrinking of the curd and expulsion of the whey.

(*c*) The acid so changes the nature of the curd as to cause "matting."

(*d*) The acid activates the pepsin of the rennet extract.

(*e*) The acid prevents the growth of putrefactive bacteria in the cheese.

3. It has been shown that *Bacterium lactis acidi* is able to form acid in the absence of the living cell.

4. The development of *Bacterium lactis acidi* is followed by the growth of another group of acid-forming bacteria, the *Bacillus Bulgaricus* group. They reach numbers comparable with those of the first group, reaching their maximum numbers within the first month of the ripening. Since they develop after the fermentation of the sugar, they must have some other source of carbon and of energy than milk sugar.

5. It is probable that coccus forms are constantly found in large numbers in Cheddar cheese.

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